The anti-amyloidogenic chaperone DNAJB6 and its interaction with other chaperones

Abstract

In order to maintain proteins' functionality their native folding state must be preserved. Chaperones fold other proteins and DNAJ-chaperones may collaborate with Hsc70 chaperones to prevent aggregation of aggregation-prone peptides such as polyQ and Aβ42, which form fibrils in currently incurable diseases as Huntington's and Alzheimer's, respectively. Fibril formation of polyQ and Aβ42 is detected by ThT fluorescence measurements over time and halftime values ($t_{1/2}$ values) of fibril formation can be calculated and used to determine how chaperones can suppress fibril formation which is observed as increased $t_{1/2}$ value. Here we observed that there is an enhancement of the DNAJB6 suppression of fibril formation by Aβ42 in the trimeric chaperone system with DNAJB6, Hsc70, Hsp110 and ATP. The increased $t_{1/2}$ values observed with the trimeric chaperone system is dependent on ATP-hydrolysis since the effect was reversed (decreased $t_{1/2}$ values) in absence of Hsp110, the nucleotide-exchange factor. Moreover, the effects were different with ATP, ADP or a non-hydrolyzable ATP-analogue. A possible interpretation is that the observed enhancement here, for the first time with DNAJB6 in the trimeric chaperone system, is the result of a continuous capture of Aβ42 oligomers, which otherwise function as nuclei for fibril formation, and continuous release as Aβ42 oligomers under ATP-hydrolysis.

Populärvetenskaplig sammanfattning

Ett protein måste upprätthålla sin tredimensionella struktur för att bevara sin funktion. De flesta proteiner veckas inte spontant till sin fungerande konformation och med tiden veckas de ut. Därför behöver kroppens celler ett system som främjar korrekt veckning och ser till att de bevarar denna veckade form. Proteiner som har denna uppgift kallas chaperoner. Chaperoner binder till och veckar andra proteiner samt samverkar i ett komplext nätverk för att bibehålla cellers proteostas. Många chaperoner är så kallade Heat Shock Proteins (Hsps), däribland DNAJ-proteiner som främst binder till felveckade proteiner och Hsp70-proteiner som binder och veckar andra proteiner.

Chaperoner förhindrar bland annat skadlig ackumulering av aggregationsbenägna peptider såsom polyQ and Aβ42. Aggregering och därpå fibrillering av polyQ och Aβ42 är associerade med Huntingtons respektive Alzheimers sjukdom, vilka båda är neurodegenerativa sjukdomar som angriper och orsakar skador på nervsystemet. I tidigare studier har man observerat att vissa Hsp70-proteiner samarbetar med DNAJ-proteiner för att dämpa sådan skadlig bildning av fibriller. Chaperonerna som användes i studien var Hsc70 (ett Hsp70-protein) och DNAJB1 (ett DNAJ-protein) samt Hsp110 som är en så kallad nucleotide exchange factor (NEF).

I tidigare forskning på Lunds Universitet, vid avdelningen för biokemi och strukturbiologi, har det visat sig att DNAJB6, ett DNAJ-protein som är likt men ändå olikt DNAJB1, verkar vara specialiserat på att binda just de former av polyQ och Aβ42, som kallas oligomerer. Det är oligomererna som orsakar sjukdom. De binds starkt till en S/T-rik region i DNAJB6, vilket är en region som saknas i DNAJB1. Baserat på detta var syftet med vårt projekt att undersöka om DNAJB6, i likhet med DNAJB1, interagerar med ett system av Hsc70 och Hsp110. Är det så att DNAJB6 kan lämna över ett substrat, som polyQ eller Aβ42, till Hsc70?

En förenkling av händelseförloppet då monomera peptider slutligen bildar fibriller kan beskrivas som en reaktion där monomerer initialt kolliderar och alltså bildar oligomerer. Dessa oligomerer är mycket skadliga för celler och sätter fart på reaktionen. Nästa fas kallas förlängning (elongation) eftersom fler monomerer nu binder in och oligomererna börjar då närma sig formen av en fibrill. När fibriller väl har bildats kan bildningen av ännu flera farliga oligomerer katalyseras på fibrillernas yta. Att fibrillerna själva fragmenteras ökar antalet fibriller ytterligare, vilket förvärrar situationen. Därför skulle det vara viktigt om DNAJB6 kan hindra och oskadliggöra de allra första oligomererna som bildas.

För att undersöka hur chaperonerna fungerar användes en metod som kallas ThT-mätning. ThT är en fluorescerande molekyl och bildningen av fibriller mäts som ThT fluorescensmätning över tid. En ökning i fluorescensen tyder på bildning av fibriller tills ett maximal-värde uppnås. Ett värde beräknades för halva tiden innan maximal-värdet uppnås (ett $t_{1/2}$ värde). Chaperonernas förmåga av att dämpa fibrilleringen kan då mätas som ett ökat $t_{1/2}$ värde.

Vi observerade att DNAJB6 kan dämpa bildning av fibriller redan vid mycket låga mängder i förhållande till aggregationsbenägna peptider. Vid förhållandet 1:0.01 Aβ42 till DNAJB6 fanns en tydlig ökning av $t_{1/2}$ värdena. En annan observation var att värdena för $t_{1/2}$ ökar kraftigt vid tillsats av DNAJB6, Hsc70 och Hsp110, men i frånvaro av Hsp110 (och ATP) blir värdena för $t_{1/2}$ mycket lägre. Detta pekar på att det trimera chaperonesystemet bestående av DNAJB6, Hsc70 och Hsp110 är beroende av ATP hydrolys. Detta tyder på en interaktion mellan DNAJB6 och systemet av Hsc70/Hsp110/ATP där substratet kan överlämnas från DNAJB6 till Hsc70.

Det dimera systemet av DNAJB6 och Hsc70 i närvaro av ATP gav noterbart låga värden, mycket lägre än när chaperonerna arbetade självständigt. Vår hypotes är att i avsaknad av ATP-hydrolys, det vill säga i frånvaro av Hsp110 och ATP, så lämnar DNAJB6 över de uppfångade oligomererna till Hsc70 som sedan släpper ut dem igen. Däremot när Hsp110 och ATP också är närvarande, sker ATP-hydrolys och Hsc70 löser då upp de skadliga oligomerer till ofarliga monomerer.

Svaret på vår huvudsakliga fråga: ”Kan DNAJB6 lämna över substrat, som Aβ42, till Hsc70” är, kort sagt, ”ja”. Åtminstone med Aβ42 och åtminstone under de testade förhållandena.

Table of contents

Abbreviations

aa	- Amino acids
Aβ	- Amyloid-β peptide
AD	- Alzheimer's Disease
HD	- Huntington's Disease
HPD motif	- Histidine-proline-aspartate motif in DNAJB6
HSF-1	- Heat shock factor protein 1
HSP	- Heat-shock protein
Htt	- Huntingtin protein
HttEx1	- Huntingtin exon-1
LGMD1D	- Limb Girdle Muscle Dystrophy type 1D
mHtt	- mutant Htt with prolonged Q stretch
NEF	- Nucleotide Exchange Factor
PolyQ	- Polyglutamine peptide
Q or Gln	- Glutamine
ThT	- Thioflavin T

1.0 Introduction

The proteins called chaperones are involved in the folding and remodelling of proteins or peptides (1). Chaperones can be found in every human cell and they take part in the synthesis and degradation of proteins in cells. Consequently, chaperones maintain the protein homeostasis (proteostasis), preventing the body from negative effects of aging and developing degenerative diseases (1). Degenerative diseases are when cells, tissues or organs degenerates or stop working. Huntington's and Alzheimer's are degenerative diseases and are caused by misfolded proteins in the neurons (2). There is no cure for Alzheimer's disease nor the rare Huntington's disease. However, studies of chaperones targeting the disease-causing peptides Aβ and polyQ in AD and HD, respectively, might inspire a way to suppress the fibril formation of these peptides, or even dissolve insoluble fibrils into soluble peptides (1). It has been reported that some DNAJs can suppress fibril formation and together with the other two types of chaperones Hsp70 and Hsp110 they can suppress fibril formation of polyQ and even dissolve the fibrils. However, further research of this cooperation of chaperones is suggested and therefore this thesis will examine the activity of the trimeric chaperone system of DNAJB6, Hsc70 and Hsp110, with a focus on DNAJB6.

1.1 Background

Most of the human proteins not only need to be folded during synthesis but are also dependent of maintaining their 3D-structure throughout their lifetime (1). The protein folding chaperones play a key role in this maintenance. Chaperones are proteins that fold or remodel other proteins, but chaperones also include proteins that in some way assist a protein folding chaperone, such as carrying a peptide and handing it to a folding chaperone (1). These are called foldases and holdases, respectively. Chaperones are continuously expressed since one of their objects is to maintain the protein homeostasis, but they can also be upregulated when cells are exposed of proteotoxic stress such as heat shock (2). The first chaperones to be discovered were the heat-shock-induced chaperones (Hsps) (2). Heat shock activates the heat shock factor 1 (HSF-1) which upregulates the expression of several chaperones (2). In this following background the folding of proteins well be described further as well as a more detailed description of the chaperones relevant of this project.

1.1.1 Folding of proteins

There are more than 25,000 different proteins important to the function of the human body (1). The role of a protein is to a large extent determined by their 3D-structure; thus, it is of great importance to keep the proteins in their right conformation (2). Proteins' native conformation, i.d. its functional tertiary structure, strives for both conformational flexibility and thermodynamic stability (1). If a protein obtains the highest flexibility, it cannot be thermodynamically stable and vice versa. In the structure of any protein, these two factors have to be compromised.

As a nascent protein is folded, it starts from a very unfavourable state of high free energy and reach the native state at a lower energy state, through making new intramolecular contacts (1). The states in between the unfolded to the native state are called folding intermediates. There are a lot of other possible conformations beside the native state and folding intermediates, such as partially folded states, amorphous aggregates, hydrophobic oligomers and amyloid fibrils (1). Amorphous aggregates, oligomers and amyloid fibrils are energetically favourable. These new intermolecular contacts with high free energy barriers are so called kinetically trapped. In a native state, the protein is more flexible and thus less thermodynamic stable and favourable than amyloid fibrils (Fig. 1).

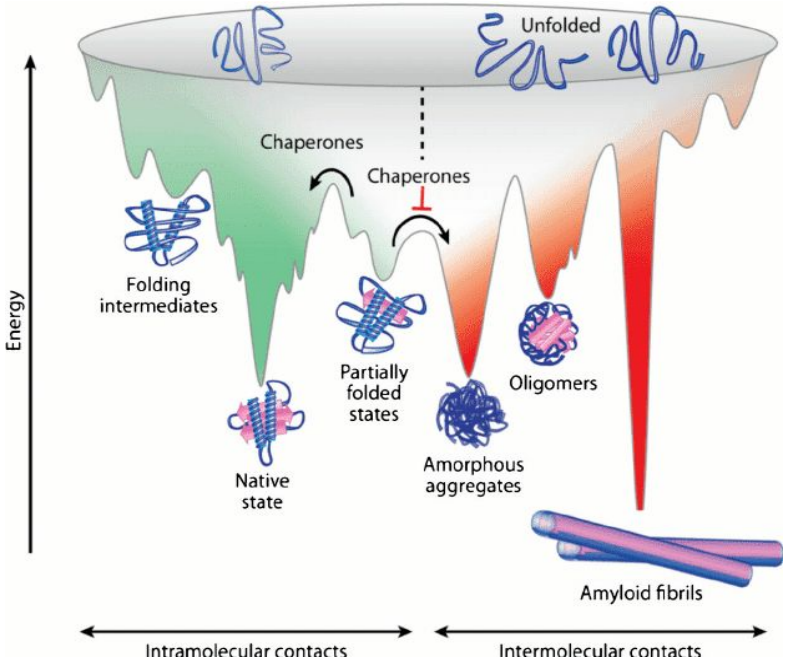


Fig. 1. The energy landscape of protein folding and misfolding (1). The conformation of lowest energy is the most stable, but the least flexible, such as amyloid fibrils.

Exceedingly few of the proteins in the human body can spontaneously fold themselves to their native state, making the proteins susceptible to misfolding (1). The hydrophobic amino acids (aa) will then be exposed to the solvent. Consequently, these residues can form intermolecular interactions, making it feasible to aggregate, especially regarding large proteins with complex structure. Also, peptides, which are shorter than proteins, can aggregate and possibly form fibrils. This is the case of the polyQ peptide in HD and Aβ peptide in AD.

1.1.2 Amyloid fibrils

In the following I will describe how it has been recently shown that the chaperone DNAJB6, which is investigated in my project, plays an important role suppressing the formation of amyloid fibrils (2). Amyloid fibrils are associated with a number of neurodegenerative diseases, as for example HD and AD. There are about 30 peptides or proteins causing diseases as they form amyloid fibrils and most of these diseases are currently incurable, such as AD

and HD. HD and AD patients can be offered only symptom-reducing treatments. The disease-causing peptide is Aβ in AD and HttEx1 in HD. Amyloid fibrils consist of β-strands that are stacked perpendicular to the fibril axis with hydrogen bonding with the polypeptide backbone. All proteins are capable of forming aggregates, at least under certain environmental conditions, but not all amyloids are deleterious. The most aggregation-prone aa sequences are the ones that stabilize the amyloid fibrils.

The amyloid fibrils form by primary nucleation followed by elongation and the secondary nucleation. The primary nucleation is a process which is dependent on the monomer concentration, such as for example the joining of two monomeric proteins/peptides. As monomeric proteins/peptides bind to the ends of the fibrils it is elongated. The secondary nucleation is fragmentation and self-catalytic fibrillation and the process is dependent on both the monomer concentration and on the presence of already formed fibril surfaces, which catalyse the process and initiate the formation of new fibrils (Fig. 2).

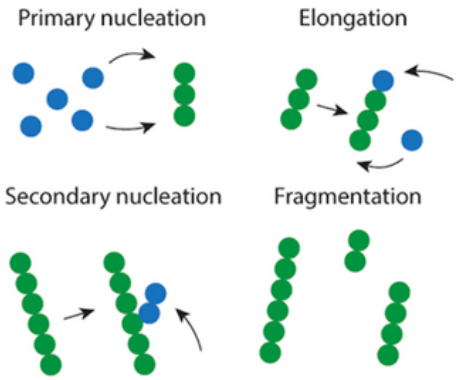


Fig. 2. A schematic figure of how amyloid fibrils are formed (3). The primary nucleation is when the monomers associate in solution. Elongation is when monomers are added at the ends of a fibril. Amyloid fibrils can be divided into smaller fragments, creating more fibrils to be elongated. Fibrils can also catalyse association of monomers on their surface.

In a closed system, the fibril formation with time can be followed and visualized in the shape of a sigmoidal curve, i.e. a curve in the shape of the letter S. At the beginning of the reaction, the solution mostly consists of monomers. However, there are also a few nuclei, oligomers and fibrils present initially. The lag phase follows by the growth phase. This is where the reaction gains speed exponentially as the fibrils elongates and form new fibrils by the two types of secondary nucleation. Finally, as the reaction reaches its plateau, most monomers are stacked in the amyloid fibrils and the reaction is then in a steady state. This is a simplification of the complex self-assembly process of amyloid fibril formation (Fig. 3).

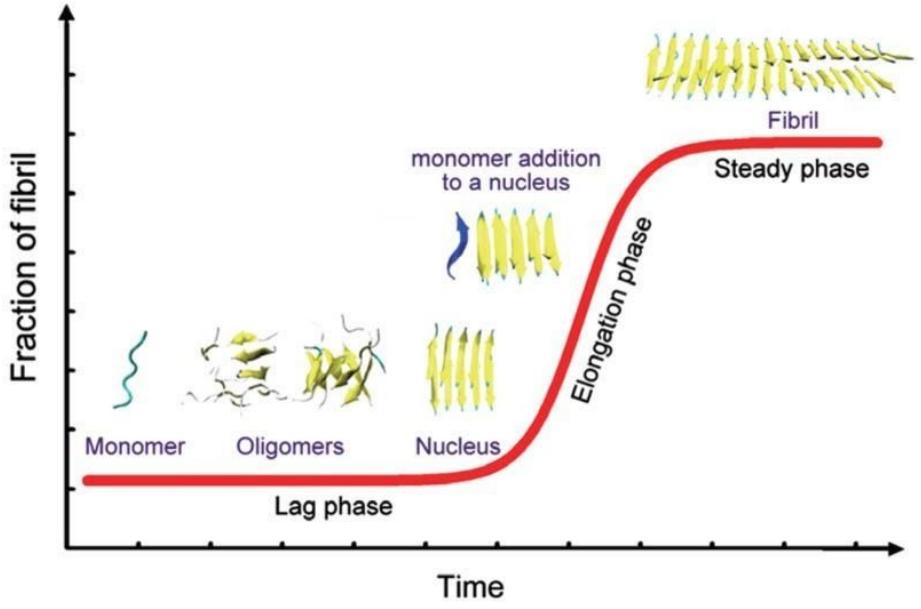


Fig. 3. The structure and kinetics of amyloid fibril formation (4). At the lag phase of the sigmoidal curve, the most common species is the monomeric protein/peptide. Over time, a couple of nuclei, oligomers and fibrils are formed. This is followed by a steep rise of fibril formation in the elongation phase as the fibrils elongate, fragment and catalyse formation of new fibrils. As the reaction goes on, most monomers are now a part of the fibril and the reaction reaches its steady state. The β-strands are perpendicular organized to the fibril axis.

1.1.3. The diseases associated with polyQ and Aβ42

Some proteins have a region of several consecutive glutamine residues. A prolongation with repeated Q residues to this polyQ stretch promotes formation of amyloid fibrils, protein aggregates and neuronal inclusions (clumps of mutant protein). This leads to neurological decline and cell death. The longer the polyQ stretch, the more prone to form amyloid fibrils it is and the severity of a polyQ disease increases with the length of the polyQ region. The prolongation is caused by a duplication of a gene. This leads to extra repeats of the CAG triplet. The repeated CAG triplet is unstable and can thereby cause even further prolongation, resulting in a more severe disease and earlier onset by each generation. PolyQ diseases are not a result of native function loss of the proteins, but a result of the deleterious effects of the expanded polyQ region.

Huntington's disease is a rare neurodegenerative polyQ disease, only 7 in 100 000 persons carries the disease, and the most common onset of HD is at 30-50 years of age. Neurodegenerative diseases as HD affects the nervous system and for HD patients the symptoms are involuntary movements but also cognitive and psychiatric disturbances. This causes dementia, depression and personality change.

Huntingtin protein (Htt) is a large (350 kDa), ubiquitous protein but mostly expressed in the nervous system. Htt usually has a stretch of 20 Q residues and if this polyQ region is expanded to more than 35 Q residues it causes disease and the Htt becomes mutated (mHtt). The proteolytic degradation of mHtt releases HttEx1 (Fig. 4) and this increases the

aggregation propensity of the polyQ stretch. Moreover, the proteasome cannot fully degrade this mHttEx1. Only the flanking residues is cut off, leaving the polyQ stretch intact which must be degraded immediately. Otherwise they will promote cytotoxic aggregation of other mHtt, HttEx1, polyQ and proteins of the PN.

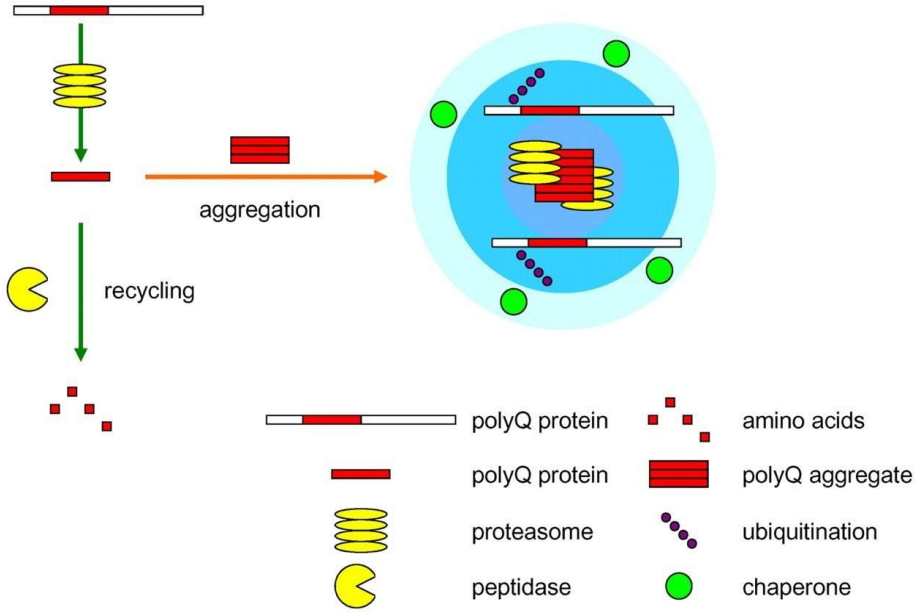


Fig. 4. Degradation of polyQ proteins and aggregation of the polyQ peptide (5). The proteasome degrades polyQ proteins, such as Htt, into peptides, and the polyQ peptide is released. These peptides can be further degraded by peptidase into separate amino acids, but if this does not happen immediately, the intact polyQ stretch will polymerize into aggregates. The surrounding chaperones and proteasomes of an aggregation is a sign of failed attempts to stop aggregation (2).

The speed of the polymerization of polyQ peptides is dependent on the formation of a nucleus, which is the primary nucleation. If the polyQ is 23 Q residues or less, four monomers are needed to form a nucleus which subsequently can form fibrils and if the polyQ is more than 26 Q residues long only one monomer is needed to form fibrils. With that said, fibrils are formed faster with longer polyQ peptides than with shorter. The speed of the fibril formation is also dependent on what kinds of regions flanking the polyQ protein which affects the second nucleation mechanism.

Alzheimer's disease is the world's most common type of dementia (60-70% of all cases) and assumingly 30 million people have the disease worldwide (6). AD is strongly related to the older population since the disease has an onset after 65 years of age in most cases (6).

AD is a neurodegenerative disease which mainly and affects the brain with symptoms like memory loss and personality changes. The symptoms gradually worsen and at some point, the disease spreads to the somatic system, causing decreased mobility and suppresses the immune system. What causes AD is not yet fully known, however, it is know that in the later stage of the disease, it is inter alia caused by extracellular Aβ plaques. These plaques are formed from

the transmembrane protein Amyloid Precursor Protein when it is proteolytically cleaved and Aβ peptides are then release in the extracellular area.

The Aβ peptides have different lengths and more or less prone to form aggregates. Aβ40 is the most common Aβ peptide, whereas Aβ42 (with the two extra C-terminal aas) is the least common one and it is more aggregation-prone than Aβ40. Imbalance in the proteostasis result in aggregation of Aβ peptide and the increased in ratio of Aβ42: Aβ40 leads to changes in the kinetics and toxicity of the aggregation.

Aβ peptide can also be found inside cells and can aggregate in different subcellular locations. The accumulation can cause synaptic dysfunction as well as disruption of the proteasome and mitochondrial functions. The formation of fibrils follows the general amyloid fibril formation process. However, the secondary nucleation mainly consists of the fibril-catalyzed nucleation.

1.1.4 Family members of the chaperones

Molecular chaperones can be found in every cell of the body and they are the protein quality control of the cells (1). Chaperones are a part of protein synthesis and degradation as well as maintaining proteostasis, i.e. keeping the proteins at a correct amount in the cells. Disrupting the proteostasis result in diseases and aging. Chaperones recognize and correct misfolded proteins, which implicates a key role in the proteostasis network (PN). The PN includes all cellular components which are part of maintaining the protein homeostasis.

Many of the chaperones are classified as heat-shock proteins (Hsps), which are upregulated when cells are exposed to heat shock (1). There are seven different families of human Hsps, whereof five are molecular chaperones: HSPA (Hsp70), HSPB (small Hsps), HSPC (Hsp90), HSPH (Hsp110) and DNAJ (Hsp40) and also the two chaperonins HSPD/E (Hsp60/Hsp10) and CCT (TRiC) (2).

Hsp70s mainly work as foldases, promoting the native state when folding nascent or unfolded proteins, which is dependent on ATP-hydrolysis (2). The members of HSPH exchanges nucleotides in Hsp70 and are also known as nucleotide exchange factors (NEFs). DNAJs or J-domain proteins (JDPs) are characterized by their J-domain of ⍺-helices (7) and have the function of holdases; they simply hold the substrates. They also transfer the substrate to HSPBs and prevent the substrate from aggregating. Moreover, JDPs enhance the ATPase activity in HSPB when interacting and, by doing so, JDPs facilitates HSPBs folding-activity of substrates since HSPB is ATP-hydrolysis dependent.

Hsp70, HSPH and DNAJ are structurally unrelated, but they all have a key role in protecting the cell by binding to substrates posing a risk of aggregation (7). In humans, there are 13 different homologues of Hsp70s, 4 homologues of Hsp110s and 41 homologues of JDPs. They can be found in every tissue in the body and their subcellular localization is in the cytosol, nucleus, membranes, ER or mitochondria. The homologues of each group differ in their substrate recognition and allosteric regulation and their adaptation to target site localization.

1.1.5 HSPA/Hsp70 – the folding machinery

Hsp70s are ubiquitous, but some members of the Hsp70 family are only expressed in specific tissues, such as the brain, heart, kidney etc. (7). They are chaperones with many tasks. Hsp70s are part of de novo protein folding, refolding of stress-denatured proteins, protein transport, membrane translocation and protein degradation (1). In order to function correctly, a complex network of different chaperons, such as JDPs and NEFs, is needed (1).

Below I will summarize current knowledge of Hsp70 structure and function according to recent review on the Hsp70 chaperone network according to which main object of Hsp70s is to keep the cellular proteins in a correct fold (8). It is substantial to preserve a protein's structure; apart from the aa sequence, it determines the function of a protein. This is why Hsp70s have a large amount of activities such as folding newly synthesized proteins, protein translocation across membrane, assembly/disassembly of protein complexes, regulation of protein activity, protection from proteolysis and cooperation with other protein folding and quality control machineries (Fig. 5). Additionally, Hsp70s also react to proteotoxic stresses. They protect the cells from negative impact through disaggregation, refolding and degrading of proteins as well as preventing protein aggregation (Fig. 5). Since Hsp70s keeps the cellular proteostasis balanced, hence mutations in the genes of the Hsp70s systems lead to numerous diseases.

Hsp70 chaperones are characterized by four domains (Fig. 6). At the N-terminal, there is a nucleotide binding domain (NBD), which is globular and binds to ATP/ADP at its central cleft. The interaction between NBD and ATP/ADP allosterically controls the coordination of the four domains. The NBD is connected by a linker to the substrate binding domain (SBD□), which consists of an eight-stranded □-sandwich where the substrate, such as a polypeptide, can bind to its hydrophobic pocket. Next to the SBD□, close to the C-terminal, is a lid of ⍺-helices (SBD⍺). The SBD is open when a substrate is binding in or leaving and closed during folding of substrate. The last domain is the tail at the C-terminal. It has different purposes depending on which type of Hsp70 it is; in nuclear Hsp70s, the tail binds to specific cofactors.

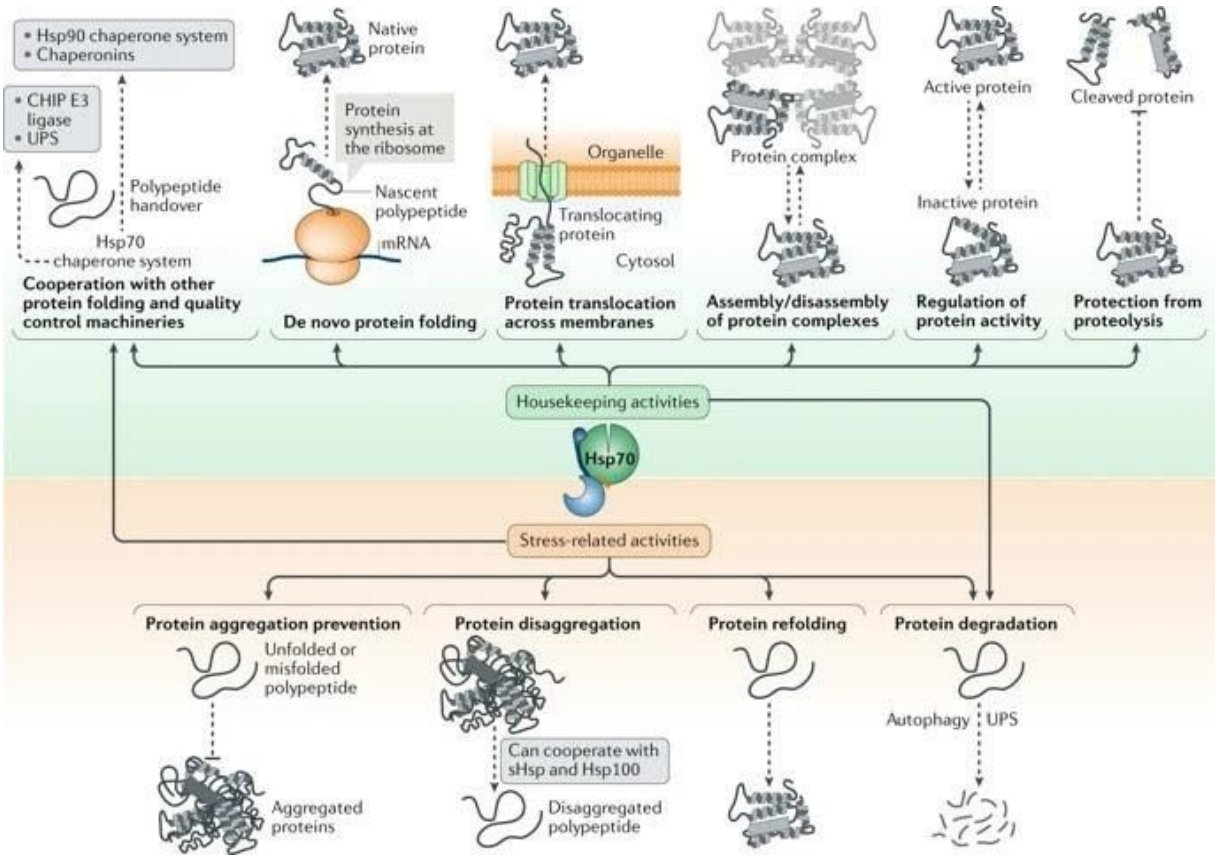


Fig. 5. All activities of Hsp70 divided in housekeeping activities and stress-related activities (8).

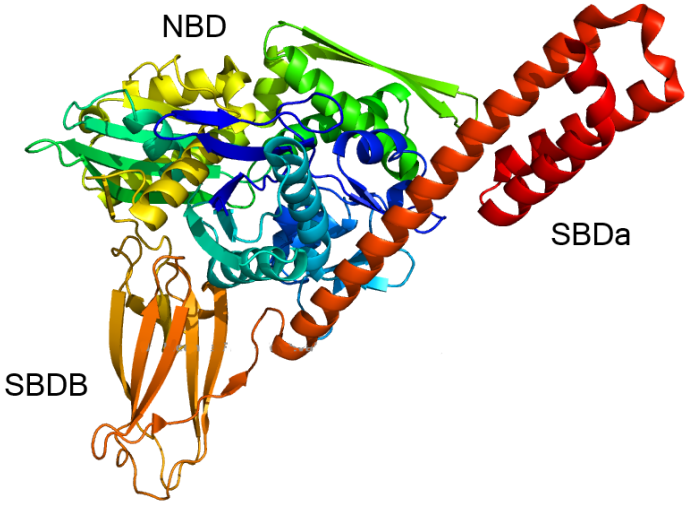


Fig. 6. DnaK (P0A6Y8), an analogue of Hsp70 (PDB ID: 4B9Q). The globular domain is NBD and the dark blue sequence is the nucleotide binding site. The orange domain of 8 β-strands is the SBDβ where the substrate binds. The red α-helices are the lid of SBDα. Image was created by me in PyMOL.

Hsp70 is dependent on the coupled reaction between the substrate binding and ATP hydrolysis to work properly. This allosteric mechanism is crucial because it enables a fast

association and a timely release of the substrate, which facilitate folding and averts aggregation. When ATP binds to the centre cleft, a small pocket is opened at the bottom NBD (Fig. 7). The linker binds to this pocket, uniting the NBD with the SBD□ which impedes the ATP hydrolysis. However, SBD□ is released from NBD as a substrate binds. As a result, ATP hydrolysis will be feasible in Hsp70, causing the α-helical lid to enclose the substrate between the SBDα and the SBD□. The enclosed substrate cannot dissociate.

The release of the of the SBD□ from NBD, though, causes the linker to leave its pocket. The NBD then looses the conformation that promotes ATPase activity. To counteract this loss of ATPase activity, preventing the substrate from dissociate that is, another allosteric mechanism occurs. The interaction between Hsp70 and the J-domain of a partner JDP hinder the linker from leaving its pocket.

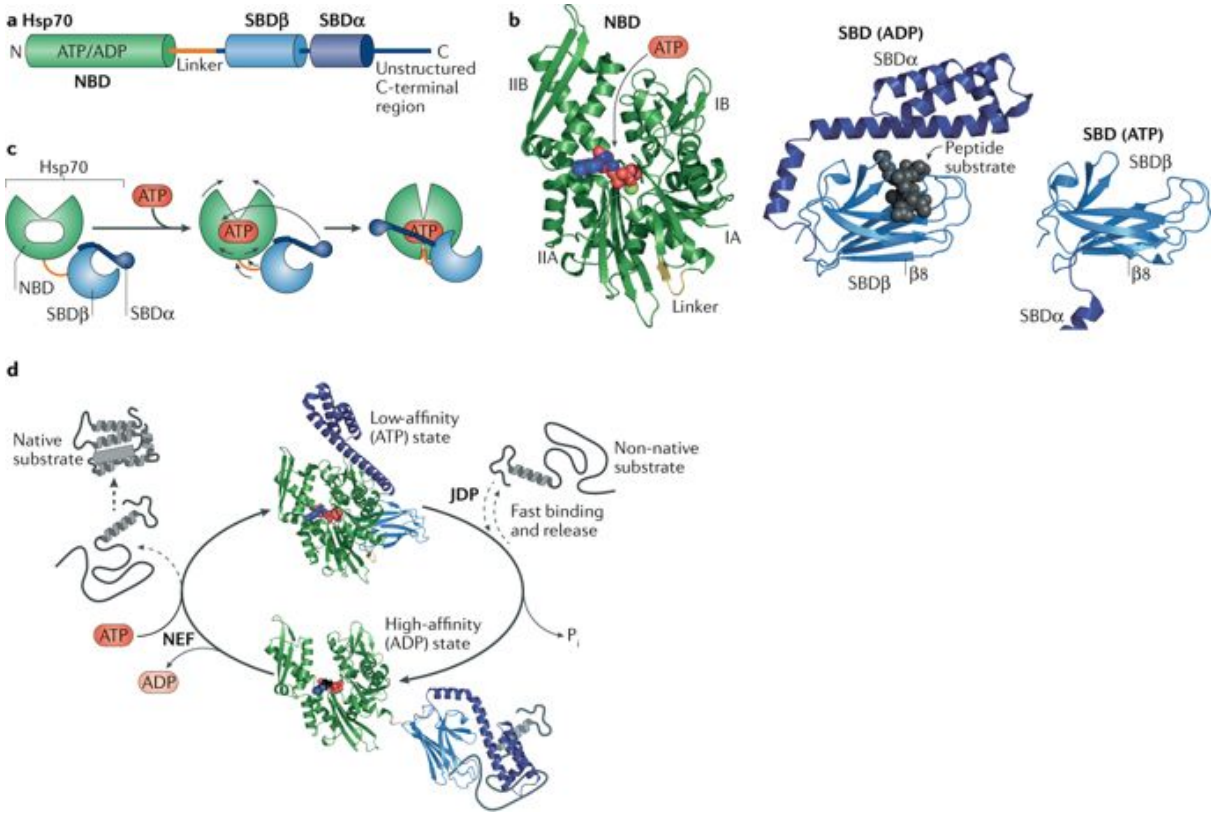


Fig. 7. The structure and mechanism of Hsp70 (8). ***a****) Order of domains in Hsp70s.* ***b****) Structure of Hsp70s: to the left is the NBD, in the middle is the closed SBD after ATP-hydrolysis and to the right is the opened SBD.* ***c****) Conformational changes of Hsp70s when interacting with ATP.* ***d****) The cycle of Hsp70s as substrates bind and release and at which point JDPs and NEFs interacts with Hsp70s.*

Binding of ATP is also involved in the release of substrates. As ATP binds to NBD, SBD□ and SBDα are separated from each other, enabling a release of the substrate as the dissociation rate is increased by a 5- to 50-fold. As the SBD□ and SBDα separates, SBD□ interacts with NBD. As a result, SBD□ undergoes a conformational change which moves □-strand 8 (in the □-sandwich) from the sheet below to the sheet above. Consequently, the substrate is more easily released as the substrate channel opens.

To elucidate how Hsp70s can recognize substrates, studies of the homolog DnaK has been performed. Most peptides binding to DnaK have a hydrophobic core of five residues and positively charged ends. These motifs occur very often in proteins, but in native proteins, they are situated in the middle of the structure. In unfolded or misfolded proteins, however, this motif is exposed, and susceptible for recognition by Hsp70s.

The substrate binding domain is conserved in Hsp70s, but its preferable substrates differ between organisms and localization. Cytosolic Hsp70s recognize aliphatic, leucine-rich peptide motifs and its ER paralogue BiP recognize aromatic peptide motifs. The orthologue HscA in *E. coli* prefers proline-rich peptides. It has also been observed that homologues from the same species localized in the same compartment, have different substrate specificity. Furthermore, it is suggested that the substrate selection is also dependent on the length of the substrate-enclosing loops and the kinetics of the SBD as well as alterations in two residues of in the SBD (M404 and A429 in DnaK and A406 and Y431 in HSPA1 and HSP8). Additionally, it has been shown that a single residue in the NBD of *S. cerevisiae* affects its activity.

As Hsp70s selectively bind to substrates exposing its hydrophobic motifs, the Hsp70s promote unfolded conformations and consequently change the free energy landscape of the substrate (8). Hsp70s changes tertiary, long-range contacts within the substrate. By disrupting these intramolecular contacts, the substrate can then form local structures. This manner could also be a part of the prevention of misfolded proteins from ending up in kinetic traps, which would cause the substrate to misfold further and/or aggregate. Hsp70 is also believed to keep the substrates in a partially unfolded state which could favour refolding. Furthermore, it is suggested that when Hsp70 interact with folded proteins, they bind at unstructured segments such as loops, linkers and tails. By doing so, the rest of the structure remain intact.

The unstructured C-terminal tail in Hsp70 is implied to be of great importance of the Hsp70s activity. When mutating this region in DnaK of *E. coli*, the refolding activity of the chaperone decreased significantly *in vitro* and its cellular thermotolerance diminished *in vivo*. Though, the function of the Hsp70s unstructured C-terminal tail is currently unknown.

1.1.6 HSPH/Hsp110 - the nucleotide exchange factors

There are four types of NEFs: GrpE type, Bag type, Armadillo type and Hsp110 type (8). GrpE is expressed in prokaryotes only, while Bag, Armadillo and Hsp110 are expressed in the cytosol of eukaryotes. The structure between these four types of NEFs differ. They have little or no homology and interact with Hsp70s differently, but all NEFs share the same function of facilitating the opening of NBD in Hsp70. They also speed up the folding of substrates in Hsp70 (7) and obstructs the released substrate from rebinding. The selectivity of NEFs is not yet known.

Since NEF types differ in interaction with Hsp70, they also differ in affinity to Hsp70 as well as their ability to induce a release of ADP and substrate. In the human cytosol, Bag is the most efficient type, followed by Hsp110 and Armadillo. The Bag family members increases

the ADP release rate up to a 100-fold, while the Armadillos increases it by a 5- to 10-fold, which suggests that these three NEFs are present in the human cells to optimize the time for Hsp70 to fold its various substrates. Though, some Hsp70s already have high ADP dissociation rates and are thereby independent of NEFs (8).

The Hsp110 types belong to the Hsp70 superfamily since they are highly homologous to Hsp70 (7). The SBD sequence and in the length of the linker is what differs between Hsp110 and Hsp70 (the link in Hsp110 is longer than the link in Hsp70). It has been observed *in vitro* that Hsp110 have a lower activity of ATP-hydrolysis than Hsp70, indicating that Hsp110s are prone to suppress aggregation, but merely holds the substrate and not refolding denatured proteins as Hsp70.

Hsp110s exist in the cytosol of the cell, but when the cell is exposed to heat stress, it transfers from the cytosol to the nucleolus (7). The nucleolus is where unfolded nuclear proteins are temporarily kept as they wait for a chaperone to fold them. A theory is that Hsp110s work as a holder of these unfolded nuclear proteins during heat stress and as the cell returns to normal conditions, Hsp110 transfers them to a protein folding chaperone.

1.1.7 DNAJ/Hsp40 - the J-domain proteins

DNAJ (Hsp40) are chaperones containing a highly conserved J-domain (7) and there are three types of DNAJ: The A-, B- and C type (Fig. 8). The A type has its J-domain at the N-terminal followed by a glycine/phenylalanine-rich site, a cysteine-rich site, which binds to zinc ions, and a C-terminal dimerization domain (8). A type and B type are very similar, the difference is that B type do not have a cysteine-rich site. The C type only has the J-domain which can be placed anywhere in the sequence and is therefore a highly diverse sub family. However, all JDPs differ in length and associated domains/motif types (8).

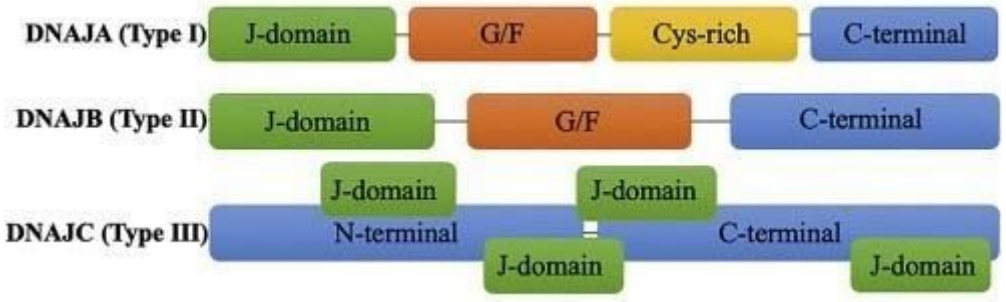


Fig. 8. The three types of DNAJ. DNAJA has a J-domain at the N-terminal followed by a glycine/phenylalanine-rich site and a cysteine-rich site. DNAJB is very similar to the A type but lacks the C-rich site. DNAJC only has a J-domain and it can be placed anywhere in the sequence (9).

The J-domain of JDPs consists of four helices (8) and all DNAJ sub families have a highly conserved histidine-proline-aspartate (HPD) motif placed in this domain (7). The interaction interface to other molecules constitutes of the evolutionary, highly conserved residues in J-domain (8). This indicates a mechanism which can be employed for all Hsp70−JDP pairs

and mutation in the J-domain causes human diseases. All DNAJ members bind to substrates as a dimer with has the structure of a V (7).

A structural model for DNAJB6 has been generated (10), which is highly similar to a recent NMR-structure of a mutational variant of DNAJB6 (11). I have used the PDB-file for the structural model of DNAJB6 (10) to generate images showing a dimer of DNAJB6 (Fig. 9). Binding of the aggregation-prone peptides is suggested to occur with the β-sheets at the monomer-monomer interface.

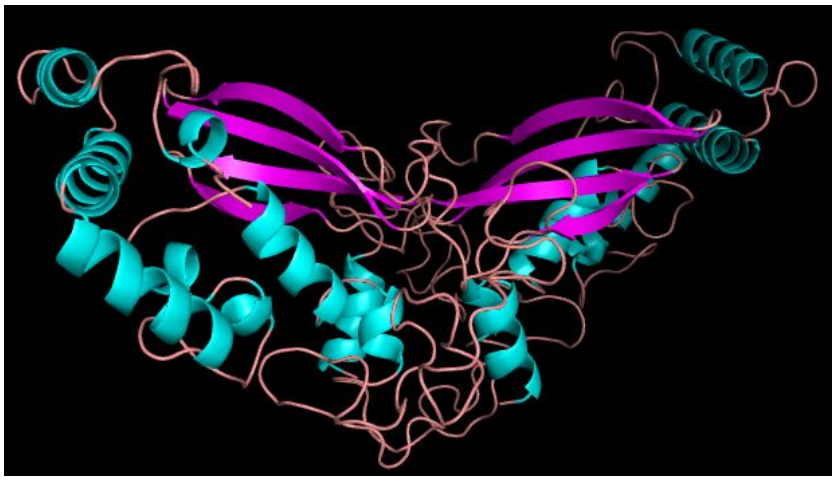


*Fig. 9.**Structure model of DNAJB6 as a dimer.** The dimer of DNAJB6 show a V-like structure occurring in many other JDPs. The β-sheets (magenta) contain the S/T.rich region, which is where the substrate binds. Image created in PyMOL.*

JDPs generally keep an unfolded protein extended as the substrate binds between the two parts of the V-shaped structure (7). It is the C-terminal domain in each of the two monomers that contains these β-strands, whereas the N-terminal domain in each of the two monomers contains the α-helices and the conserved HPD-motif, which is crucial for the interaction with Hsp70 (Fig. 10).

The J-domain, including its HPD motif, interacts with Hsp70 by forming polar connections with the NBD-SBDβ interface and by forming hydrophobic contacts with the linker-SBDβ interface (8). Hsp70 binds to the longer helices II, III and their connecting loop containing the HPD motif. The binding of HPD to Hsp70 is of utmost importance for the JDP activity. An indication of this is the loss of activity of DNAJB2 as its HPD motif is mutated to QPD (7). The motif binds to residues coupled with two allosteric mechanisms of Hsp70 which regulates ATPase activity in Hsp70 (8). It couples the substrate binding to ATP hydrolysis and efficient trapping of the substrate. In other words, this interaction facilitates hydrolysis of ATP in Hsp70 and thus also the activity of this foldase. Controlling regulation of Hsp70 activity could pose a treatment of neurodegenerative diseases and cancer, which may be of interest to drug development.

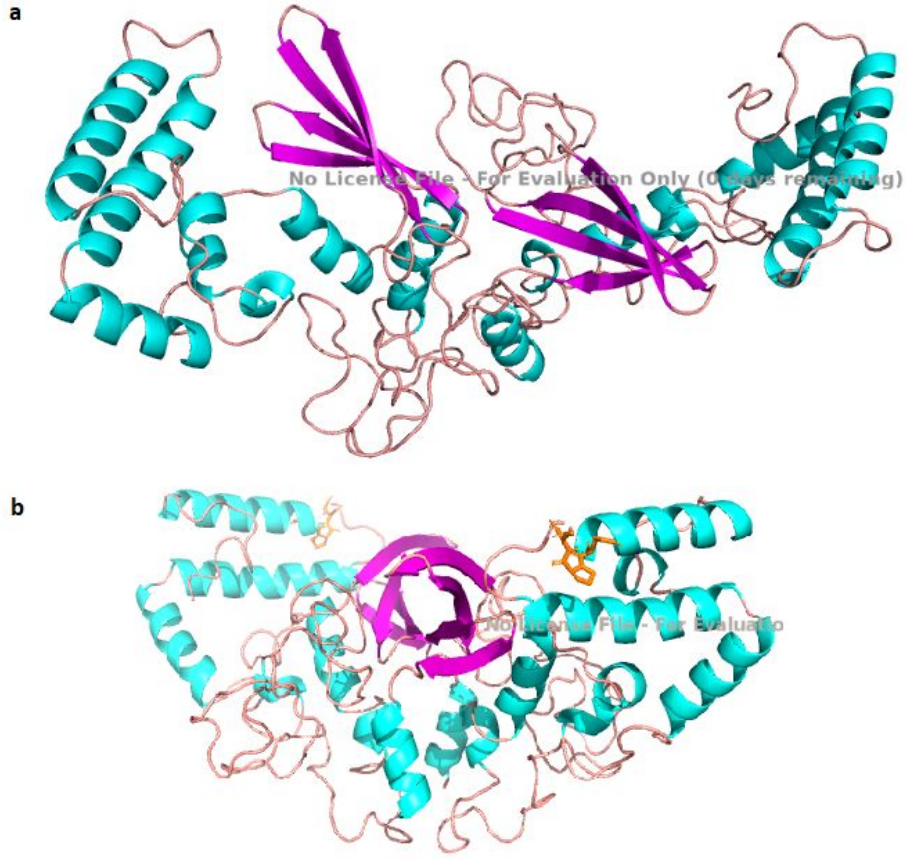


Fig. 10. In the DNAJB6 dimer the C-terminal domains face each other, and the N-terminal domains interact with Hsp70. a. The V-like dimer of DNAJB6 from above. The substrate binds to the β-sheets (magenta), keeping the substrate extended in this conformation of DNAJB6. b. Viewing the V-shape from the side. The orange residues are the HPD-motifs, which is an important site of interaction for DNAJB6 with Hsp70. The HPD-motif is situated in a loop connecting helix II and I(cyan). Image created in PyMOL.

Some JDPs also display other regions that interact with Hsp70s except the J-domain, which enables adjustment of the allosteric regulation (8). The C-terminal of most Hsp70s ends with an EEVD motif. This motif can bind to the C-terminal domain (CTD) of some JDPs which activates the function of the cooperating JDP-Hsp70 pair. Deleting the EEVD motif leads to a loss of the refolding activity of misfolded proteins whilst interacting with these specific JDPs. However, the foldase is still operational with other substrates and JDPs that do not require the activity regulator of the EEVD-CTD interaction. In short, the activity of the Hsp70-JDP machinery is regulated by different means.

Substrates bind to a JDP at its β-sheets, but it is also possible that the conserved G/F-rich linker work as an interaction site as well. Deletion or mutation in the G/F-rich domain leads to a decrease in its utility and also its affinity of partially and fully folded substrates (8). The interaction of JDPs with substrates is essential for initiation of the cycle of Hsp70. It has been reported that the number of possible Hsp70 binding sites in substrates increases by the

presence of JDPs, which is probably caused by a conformational change of substrates induced by JDPs. A theory is that, by doing so, JDPs are able to select and somewhat remodel substrates which enhances the substrates' availability.

The DNAJ proteins have various preferences of substrates. For example, human DNAJA2 prefers small aggregates (around 200-700 kDa), whereas DNAJB1 rather bind to large aggregates of 700-5,000 kDa (8). DNAJB6 and DNAJB8 both have a S/T-rich region. This region binds to polyQ and Aβ, which impedes them from forming and elongating amyloid fibrils. Of note, this function of DNAJB6 and DNAJB8 is independent of the cooperation of Hsp70s.

The purpose of DNAJs is to determine the specificity of the Hsp70s (2). It is then not surprisingly that DNAJs have 41 encoding genes, compared to Hsp70s mere 13 encoding genes (7). Some DNAJs are ubiquitously expressed, whereas some are only expressed in certain tissues, such as brain-, heart- and kidney tissue amongst many other tissues. Even if they are ubiquitously expressed the level of expression variates, as for DNAJB6. It can be found in every cell but appears in higher concentration in the brain (2). There is some variation in the subcellular localization of DNAJs as well (7). For instance, DNAJA4 is located in the membranes of brain cells, whereas DNAJB1 can be found in every cells' cytosol.

The sub family DNAJB is the second largest sub family and consists of 14 different proteins (7). Except the high homology of the J-domain in this group, DNAJB6, DNAJB7 and DNAJB8 are much alike in their C-terminal. It is not known what functions DNAJB7 and DNAJB8 have, but DNAJB6 is known to work as an anti-aggregation protein.

1.1.8 DNAJB6

Below I will summarize current knowledge of DNAJB6 structure and function according to a PhD thesis in the role of DNAJB6 was explored (2). Isoform a is the largest as isoform b is about 80 aa shorter and they also differ in aa sequence of the C-terminus. Isoform a is located in the nucleus whereas isoform b is located in the cytosol, but, after exposure of heat shock to the cell during interphase in mitosis, isoform b is transferred to the nucleus. DNAJB6 is one of the members of the B sub family that is ubiquitously expressed. However, they occur at higher amounts in the brain, especially in the hippocampus and thalamus.

DNAJB6 has many functions such as suppressing amyloid fibril formation of mHtt, promoting proteasomal degradation of keratin intermediate filaments and restrain replication of HIV-1 with help from Hsp70. It has also been observed that DNAJB6 play a role in the placental development in mice.

When short of DNAJB6, keratin intermediate filaments accumulate. Other studies have shown that breast cancer is connected to the downregulation of DNAJB6 in breast cancer cells, which subsequently promotes invasion of tumour cells. The pathology of the myopathic disease Limb Girdle Muscle Dystrophy type 1D (LGMD1D) is a mutation of the gene

expressing DNAJB6. Studies have shown that the half-life of DNAJB6 is elongated when point mutations in the G/F-rich domain (such as F89I, F93L and F96I) of DNAJB6 is introduced, which causes protein aggregation in muscle fibers. DNAJB6 accumulates with Hsc70 and HSPB8 in LGMD1D patients. It also accumulates with other proteins that typically occur in aggregates in myopathies, for example myotilin, desmin and keratin 18.

The S/T-rich region is crucial for the function of DNAJB6 (12). When substituting S/T with A, DNAJB6 gradually loses its ability to suppress fibril formation of Aβ42 peptides the more substitutions of S/T to A. Substituting 18 S/T residues appeared to not change the structure of DNAJB6, however, it nearly completely impaired the chaperone's suppression ability. The substitution affects the primary nucleation of Aβ42 peptides (12) and decreases the ability of DNAJB6 to suppress fibril formation of Aβ42 and polyQ peptides (11). This demonstrates a general role in amyloid fibril formation suppression of various aggregation-prone peptides by the S/T residues. DNAJB6 is undoubtedly an important chaperone in several processes of the cells, which is why this project will focus on DNAJB6.

1.2 This project and its aim and objective

As described above chaperones are generally important in avoiding aggregation of other proteins. During research in this laboratory over the past years it has been found that DNAJB6 is unusually efficient in suppression of fibril formation by the aggregation-prone peptides polyQ and Aβ42. For DNAJB1, a DNAJ-protein homologous to but different from DNAJB6, other researchers recently have demonstrated that it can collaborate in a trimeric chaperone system, with DNAJB1, Hsc70 and Hsp110, and completely suppress fibril formation of polyQ (13). Also, other DNAJ-proteins appear to have this ability, as part of an intricate DNAJ-protein network driving protein disaggregation (14).

Thereby, the aim of this project is to study DNAJB6 in a trimeric chaperone system of Hsc70, Hsp110 and DNAJB6. The question to be answered is: Can DNAJB6 hand over its substrate, such as polyQ or Aβ42, to Hsc70?

2.0 Materials & Methods

2.1 Materials

Proteins. The DNAJB6 (UniProt acc nr O75190_2) human chaperone protein, prepared as described in (2) and (12), was available as flash-frozen aliquots stored at minus 80 degrees. Aliquots were thawed and stored on ice at a concentration of 2 mg/ml between experiments. Two different batches were used (referred to as opt 1 and opt 5, with opt 5 used if not stated differently). The mutational variant, DNAJB6 F91L, had been prepared as DNAJB6 WT. The other two chaperone proteins used, HSPA8/Hsc70 (UniProt acc nr P11142) and Hsp110/Apg2/HSPA4 (UniProt acc nr P34932), prepared as described in (13), were a kind gift from Janine Kirstein in Berlin. The final concentration of these chaperones was 0.2μM and 0.1μM respectively, unless otherwise stated.

Peptides. The polyQ construct used constituted maltose binding protein (MBP), followed by a TEV cleavage site and a stretch of 45 or 15 Q (negative control) residues, were used with TEV protease for initiation of fibril formation as described previously (2), and was a kind gift from Ron Melki, Paris. The Aβ42, purified as described previously (12), was supplied by Sara Linse as monomers freshly isolated by size exclusion chromatography in 20 mM sodium phosphate, 0.2 mM EDTA pH 7.4, and kept on ice until used in assays.

Chemicals. ATP, ADP and ATP-analogue were sodium salts with the following product numbers A2383, A2754, A1388 and purchased from Sigma-Aldrich, St. Louis, USA. Stocks of 128 mM were prepared in buffer of H_2O, HCl, KOH (for ATP-analogue also NaPB and EDTA) and stored as aliquots at minus 20 degrees until used. Thioflavin T was obtained from Sara Linse as a 2 mM stock solution.

2.2.0 Methods

In order to follow the amyloid fibril formation *in vitro*, ThT fluorescence measurements were performed as described previously (12). The fluorescence measurements on polyQ were performed in 20 mM sodium phosphate or/and 200 mM HEPES as indicated in the results. The pH was set to 8.0 in all buffers, because DNAJB6 is most soluble at this pH. In some experiments the ion strength was increased due to presence of 150 mM NaCl or 150 mM NaCl + 5 mM $MgCl_2$., as indicated in the results. The concentrations of ThT was 10 μM throughout all experiments and the concentration of the aggregation-prone peptides was varied between experiments between 10 and 20 μM for polyQ and between 2 and 5 uM for Aβ42. The concentrations of DNAJB6 was varied, as indicated in the results, with a molar ratio of peptide to DNAJB6 between 1:0.01 and 1:0.1. The concentration of the chaperones Hsc70 and Hsp110 was 0.2 μM and 0.1 μM to be comparable with the data presented in Scior et al 2017. The concentrations of nucleotides ATP, ADP and the non-hydrolyzable ATP-analogue was 5 mM, unless otherwise stated. An example of the calculations and the performance is presented in appendix 1 as an excel sheet.

In order to estimate whether a difference in samples is significant or not, the standard error (SE) of all triplicates were calculated by the following equation:

$$SE = \frac{\sigma}{\sqrt{n}}$$

Where σ is the standard deviation, calculated by a graphing calculator, and n is the number of measurements.

2.2.1 Protein concentration determination and analysis of proteins by SDS-PAGE.

Prior to the fibril formation assays the proteins to be used were subjected to protein concentration determination by recording the absorbance at 280 nm using 1 ul aliquots and taking the average of a least 3 readings on a NanoDrop microvolume spectrophotometer (Thermo Fisher Scientific, Stockhom, Sweden). The relative molecular masses were inspected after denaturing electrophoresis in which proteins are denatured by the anionic detergent sodium-dodecyl sulphate (SDS) and then exposed to polyacrylamide gel electrophoresis (PAGE). The gels were loaded with 10 µg of a protein in each well and after separation the proteins were vizualized by CBB-staining and images recorded by a gel scanner as previously described (12).

3.0 Results

Before starting the actual ThT fluorescence assays to measure how DNAJB6 and the other chaperones suppress fibril formation by the peptides polyQ and Aβ42 I performed some initial experiments to find a suitable buffer for the measurements and to characterize the peptides and proteins that I was going to use. I will first shortly describe the results of these initial experiments. Then I will describe the results obtained with the ThT fluorescence assay and the effect of the chaperones on fibril formation by polyQ peptides (10 repeats), and Aβ42 peptides (6 repeats).

3.1 Testing the buffering capacity against ATP

Previously performed ThT fluoresence assays (e.g. Månsson et al 2018) have been routinely performed in 20 mM sodium phosphate buffer at pH 8. It had been observed in initial attempts to use the trimeric chaperone system that addition of 5 mM ATP caused huge effects in the assay, also in the controls with only the aggregation-prone peptide, with much faster aggregation. Since it is well known that the ThT assay in 20 mM sodium phosphate buffer is very dependent on the ionic strength and pH it was suspected that, since ATP is acidic, the huge effects were caused by pH changes. Therefore, I first started to directly measure, with a pH meter, to what extent there is a pH change with and without 5 mM ATP in 20 mM sodium phosphate buffer pH 8, and if other buffer systems better could resist pH changes.

The change in pH when adding 4 mM ATP were tested in the three buffers set to approximately pH 8, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer , sodium phosphate buffer (NaPB) and Tris (tris (hydroxymethyl) aminomethane) buffer at the concentrations of 20, 40, 60, 100 and 200 mM. The pH was measured before and after adding the ATP and the values are presented in Table 1.

Table 1. ***Buffering capacity of HEPES, NaPB and Tris at various concentrations.*** *Table shows pH values measured with pH meter before and after adding 4 mM.*

	Concentration	pH before ATP	pH after ATP	ΔpH
HEPES	20 mM	7.66	7.03	-0.63
	40 mM	7.66	7.33	-0.33
	60 mM	7.66	7.44	-0.22
	100 mM	7.67	7.52	-0.15
	200 mM	7.68	7.60	-0.08
NaPB	20 mM	8.15	7.03	-1.12
	40 mM	8.15	7.36	-0.79
	60 mM	8.11	7.47	-0.64
	100 mM	8.08	7.60	-0.48
	200 mM	8.03	7.72	-0.31
Tris	20 mM	7.82	3.77	-4.05
	40 mM	7.87	3.81	-4.06
	60 mM	7.87	3.93	-3.94
	100 mM	7.88	4.29	-3.59
	200 mM	7.94	5.11	-2.83

The results showed that Tris had a very low buffering capacity at all tested concentrations of 20; 40; 60; 100; 200 mM compared to NaPB and HEPES. Even at the highest concentration of 200 mM the pH value decreased by 2.83 units. The buffering capacity to acidity decreased with lower concentrations of Tris. At the concentrations below 200 mM the pH change is about -4 pH units. The lowering of buffering capacity with decreasing buffer concentration is also seen in NaPB and HEPES-buffer, as the ΔpH gradually increases as the buffer concentration decreases. In 20 mM NaPB the pH value decreased with 1.12 units while in the higher concentration of 200 mM the pH value decreased with 0.31 units. With HEPES, however, the pH lowered with less than 1 unit at all tested concentrations, with the highest change being -0.63. Of all tested buffers with varying concentrations, 200 mM HEPES displayed the least change in pH by -0.08 units. We concluded from the pH meter measurements that indeed addition of 4 mM ATP to 20 mM NAPB give a pH change of more than 1 pH unit. We decided to use the well-buffered system with 200 mM HEPES alongside with 20 mM sodium phosphate to explore how the ThT fluorescence assay behaved in different buffers.

3.2 Analyis of proteins used by SDS-PAGE

In order to inspect and analyze the molecular masses of the proteins to be used the proteins were analysed by denaturing electrophoresis (SDS-PAGE) and stained with CBB. By SDS-PAGE can also be an indicator of the purity of the protein. In the assays to be used fibril formation by polyQ is initialized by releasing the polyQ peptide by cleavage from a fusion protein (MBP-Q45) that contains cleavage site for the protease TEV (Tobacco Etch Virus).

This process could also be visualized by SDS-PAGE by loading MBP-Q45 with or without prior cleavage. Aliquots with 10 µg of each protein, or varying amounts, were loaded (Hsp70, MBP-Q45, DNAJB6 WT and DNAJB6 mutant ST18A), and a gradient of the amount of loaded protein case of DNAJB6 WT and MBP-Q45. Gel images obtained after CBB-staining and scanning are presented in Fig. 11.

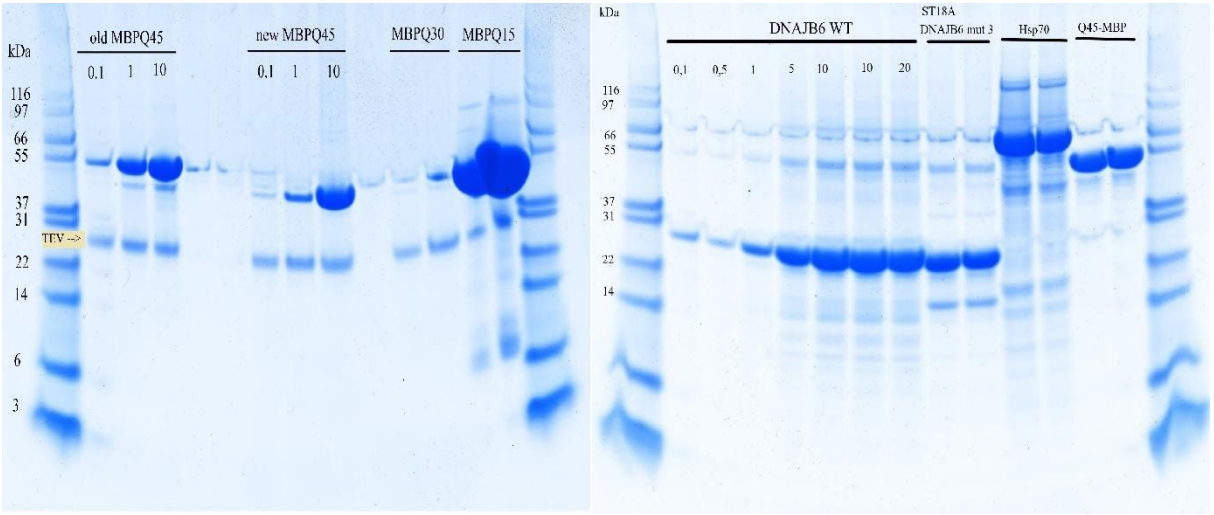


Fig. 11. ***Images of CBB-stained SDS-PAGE gels showing several of the proteins used in study. A)*** *Variants of MBP-polyQ fusion proteins loaded after adding TEV-protease, from left to right: and old and a new preparation of MBP-Q45 (0.1, 1 and 10 ug), and unknown amounts of MBP-Q30 and MBP-Q15. The band representing TEV is marked (NB. TEV and DNAJB6 has approximately the same mass).* ***B)*** *from left to right: DNAJB6 WT (1, 5, 10, 50 and 100 ug), DNAJB6 mutant ST18A (duplicates 10 ug), Hsc70 (duplicates 10ug), MBP-Q45(duplicates 10 ug) without TEV-cleavage. The running buffer used was MES and the edge lanes of both gels show Mark-12 molecular weight standard.*

By comparing the protein bands with the bands visible in the reference all masses are in the expected ranges; the mass of Hsp70 was estimated to 67 kDa and of MBP-Q45 to 52 kDa and the mass of the monomer subunit of DNAJB6 is 27 kDa, for both DNAJB6 WT and mutational variant ST18A (not used in the study but included for comparison). There is also a dimer band seen at approximately 54 kDa. Increased amounts of protein loaded shows an increased staining of the band for DNAJB6 and MBPQ45, indicating that the CBB-staining of the bands is indeed proportional to the amount of protein loaded, as should be expected. In the well of Hsp70, there are a lot of other bands visible suggesting that there are some contaminating proteins present.

The addition of TEV to the MBP-Q45 (new) displayed a decrease in mass with about 6 kDa to 46 kDa in response to TEV-cleavage, as expected. This illustrates how, in the ThT fluorescence assay, release from the MBP-Q45 fusion protein of the polyQ peptide with 45 Q-residues is initiating fibril formation when TEV-protease is added just prior to starting to record the ThT fluorescence. An older MBP-Q45 preparation, stored a long time in freezer and not used in this study, was however NOT cleaved, perhaps it already had formed fibrils.

3.3 Effects of chaperones on fibril formation by polyQ

In order to follow the amyloid fibril formation *in vitro*, fluorescence spectroscopy was performed. As molecules absorbs a photon, they reach an excited state, which is a higher energy state than their ground state (2). The molecule prefers the ground state of lower energy. Therefore, the photon is released, emitting light with lower energy than the absorbed light. Lower energy means longer wavelength. The amount of a molecule can be estimated by measuring the intensity of the emitted light.

Thioflavin T (ThT) is a fluorophore, which means that it is a fluorescent chemical compound. It binds to amyloid fibrils (2). When ThT binds to fibrils, the maximum excitation and emission shifts from 385 nm and 445 nm, respectively, in the unbound state to 450 nm to 480 nm. To measure the amyloid fibril formation the sample is excited with 440 nm and the light emitted is detected at 480 nm. An increase in fluorescence corresponds to fibril formation.

A typical curve from an ThT fluorescence measurement on the fibril formation by polyQ or Aβ42 has the shape of a sigmoidal curve as seen in Figure 1. The curve initially has a flat lag phase, when oligomers and nuclei are formed, followed by a phase with a steep rise in the fluorescence curve when fibrils form from nuclei and finally a plateau when fibrils have formed.

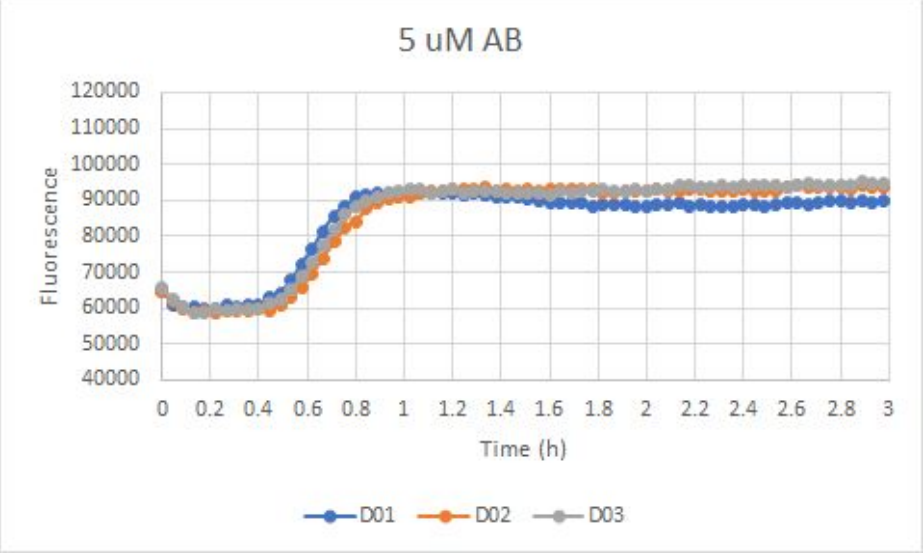


Fig. 12. ***ThT fluorescence measurement showing fibril formation by of 5 µM Aβ42****. The data points form a sigmoidal curve from which a half time (t1/2-value) can be calculated from the triplicate samples (blue, orange, grey). Data taken from experiment from 200427.*

In order to compare curves with one another a half time value (t1/2-value) is calculated from the curve. Half time is the time when the fluorescence has reached half of its steady state fluorescence value. In the following I will present my results in tables with such t1/2 values determined, with the corresponding fluorescence curves supplied in the Appendix. An overview of all experiment is provided in the Appendix Table A1. Usually triplicate samples

are used to determine an average t1/2-value but in the beginning, I also used singlicate and duplicate samples to save material and get an initial overview.

3.3.1 Effect of ion strength on fibril formation of polyQ and suppression of fibril formation by DNAJB6

The first experiments polyQ was added in NaPB, HEPES or NaPB/HEPES at different ion strengths (Table 2) in order to observe how different ion strengths affect the rate of fibril formation of polyQ.

Table 2. ***PolyQ and DNAJB6 in NaPB and/or HEPES buffer at different ion strengths.*** *The average $t_{1/2}$ from the fluorescence measurements with 10 µM polyQ in NaPB pH 8 at different ion strengths ± 200 mM HEPES pH 8. Concentrations of salts: NaCl 150 mM, Mg Cl_2 5 mM. From experiment 200210 and 200218.*
'=singlicates
''=duplicates
SE=Standard Error (-=not calculated)

Buffer	No HEPES	HEPES	0.1 µM DNAJB6	1 µM DNAJB6
NaPB	5.0 h	5.0 h''	14 h''	43 h''
NaPB/NaCl	4.0 h''	7.0 h''		
NaPB/NaCl/MgCl,	6.0 h''	7.0 h''		

The $t_{1/2}$ values are roughly the same in all conditions shown in Table2. The addition of 0.1 µM DNAJB6 increases the $t_{1/2}$ value to 9 h and 1 µM DNAJB6 increases the half time to 43 h, which is almost a 10-fold longer half time. We concluded that the time it takes for 10 µM polyQ to form fibrils is not changing much depending on buffer and ionic strength and that DNAJB6 can suppress fibril formation, as expected from previous data (2). Here at a molar ratio of polyQ to DNAJB6 that is 1:0.1 and 1:0.01 the half time is 8.6-fold and 2.8-fold, respectively.

3.3.2 Effect of DNAJB6 and Hsc70/Hsp110/ATP on fibril formation of polyQ

The effect of DNAJB6 alone and of the trimeric chaperone system (DNAJB6/Hsc70/Hsp110 and ATP) on fibril formation of polyQ was tested in HEPES buffer at different ion strengths, with the results presented in Table 3.

Table 3. ***PolyQ and DNAJB6 in absence and presence of Hsc70/Hsp110/ATP in HEPES buffer.*** *The average $t_{1/2}$ from the fluorescence measurements with 10 µM polyQ in 200 mM HEPES buffer pH 8 at different ion strengths and with or without 0.01 µM or 0.1 µM of DNAJB6. Concentrations of salts: NaCl 150 mM, Mg Cl_2 5 mM. The presence of Hsc70/Hsp110/ATP is indicated in the table by the abbreviation HHA.*
'=singlicates
''=duplicates
'''=more than three $t_{1/2}$ values

HEPES buffer	No DNAJB6	0.1 µM DNAJB6	1 µM DNAJB6
No salts	6.5 h'''	8.0 h''	18 h''
NaCl	6.0 h''		
NaCl/$MgCl_2$	8.0 h'''	10 h'	23 h'''
HHA	8.5 h''	8.0 h'	23 h'
NaCl/$MgCl_2$+ HHA	8.0 h'''	11 h'	21 h'''

In Table 3 the average half time of 10 µM polyQ in 200 mM HEPES is 6.5 h, with no major difference depending on the buffer and ionic strength. DNAJB6 increases the $t_{1/2}$ to 8-10 h at 0.1 µM and to 18-23 h at 1 µM. i.e. nearly 3-fold. Thus, DNAJB6 suppressed the fibril formation of polyQ in 200 mM HEPES buffer, but the effect was somewhat less pronounced that in Table 2 in 20 mM sodium phosphate buffer. The presence of Hsc70/Hsp110/ATP gave essentially no difference in suppression compared to DNAJB6 alone (the t1/2-value was 1.3-fold compared to DNAJB6 alone in absence if salts, and 0.9-fold in presence of salts).

3.3.3 Effect and ATP-dependency of the trimeric chaperone system with DNAJB6 and/Hsc70/Hsp110 on fibril formation of polyQ using ATP, ADP and ATP-analogue in triplicate samples

Since any effects of the trimeric chaperone system (DNAJB6/Hsc70/Hsp110/ATP) are expected to be ATP-dependent we also performed experiments in which ATP was replaced with ADP or a non-hydrolyzable ATP-analogue. Since it was very unclear from the data in Table 3 whether there is an effect of Hsc70/Hsp110/ATP all measurements included triplicate samples to average out possible effects of experimental errors. This is presented in Table 4.

Table 4. ***PolyQ with Hsc70 and Hsp110 in absence or presence of DNAJB6 and ATP, ADP or a non-hydrolyzable ATP-analogue.*** *The half times of 10 µM polyQ in HEPES/NaCl/$MgCl_2$ buffer. Hsp70 and Hsp110 is present in all samples and all values are derived from triplicate samples. Data from 200219, 200220, 200224, 200227, 200303 and 200311.*

Nucleotides	No DNAJB6	SE	1 µM DNAJB6	SE	Fold-change by DNAJB6
ATP	8.0 h	0.4	21 h	1.4	2.6
ADP	6.5 h	0.4	13 h	0.2	2.0
ATP-analogue	6.5 h	1.1	19 h	3.8	2.9

With ATP present the t1/2-value is 8 h, meaning that, as previously observed in Table 3, the presence of Hsc70/Hsp110/ATP only, without DNAJB6 has no effect in suppressing fibril formation. Adding 1 µM DNAJB6 increases the t1/2-value to 21 corresponding to a 2.6-fold increase, as previously observed in Table 3. When replacing ATP with ADP or non-hydrolyzable ATP-analogue the half times slightly decreased in all samples, with or without DNAJB6. Repeated measurements were performed in absence and presence of DNAJB6 and various combinations of Hsc70, Hsp110 and ATP. The $t_{1/2}$ from these experiments are shown in Table 5.

Table 5. ***PolyQ in absence and presence of DNAJB6 and various combinations of Hsc70, Hsp110 and ATP.*** *Averages of half times of 10 µM polyQ in HEPES/NaCl/MgCl2 buffer at pH 8 and all values are derived from triplicate samples. Data from 200219, 200220, 200224, 200225, 200227, 200303 and 200311.*

	No DNAJB6	SE	1 µM DNAJB6	SE	Fold-change by DNAJB6
Hsc70/Hsp110/ATP	8.0 h	0.4	21 h	1.4	2.6
Hsc70/ATP	8.5 h	0.6	23 h	2.3	2.7
Hsc70/Hsp110	9.5 h	0.6	27 h	0.5	2.8

There were no or very small differences seen when any of Hsc70, Hsp110 and ATP were excluded. The $t_{1/2}$ –value for 10 µM polyQ was 8.0-9.5 h and with 1 µM DNAJB6 present, all $t_{1/2}$ are approximately 3-folded. Thus, under the currently investigated conditions, there appears to be essentially no effect of Hsc70/Hsp110/ATP on the fibril formation by polyQ, whereas DNAJB6 only suppressed the fibril formation with a 3-fold increase in the t1/2-value. There was also no effect of Hsc70/Hsp110/ATP in an additional experiment when DNAJB6 was compared to a mutational variant. DNAJB6 F91l, as shown in Table 6.

Table 6. ***PolyQ with DNAJB6 WT and DNAJB6 F91L in absence and presence of Hsc70/Hsp110/ATP.*** *Averages of half times of 10 µM polyQ in HEPES/NaCl/$MgCl_2$ buffer at pH 8. Data from 200219, 200220, 200224, 200227, 200303 and 200311.*

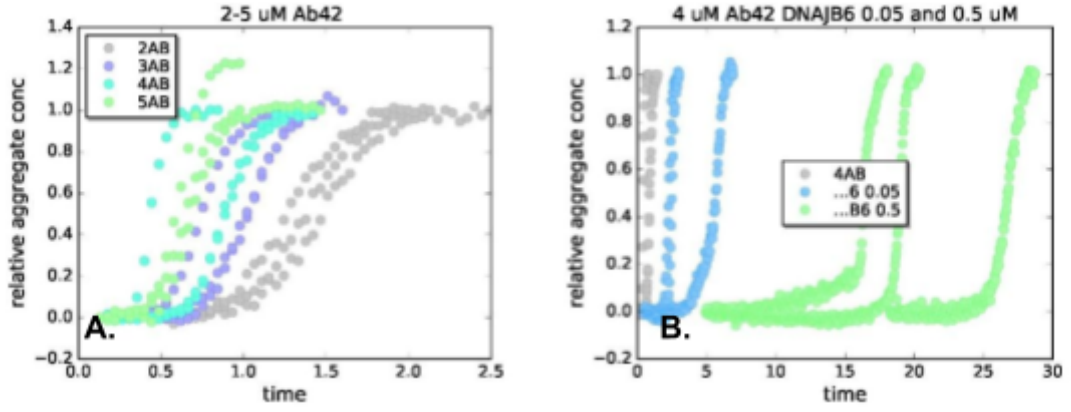
	No DNAJB6	SE	1 µM DNAJB6	SE	1 µM F91L	SE
No Hsc70/Hsp110/ATP	8.0 h	0.4	23 h	2.0	24 h	1.9
With Hsc70/Hsp110/ATP	8.0 h	0.4	21 h	1.4	25 h	2.1

According to the $t_{1/2}$ values of 10 µM polyQ in Table 6 there is no major difference between DNAJB6 and F91L. However, in the samples containing Hsp70/Hsp110/ATP as well, the $t_{1/2}$ is 4 hours more with F91L instead of DNAJB6.

3.4 Effect of chaperones on fibril formation by Aβ42

In order to further study the behaviour of DNAJB6 together with the other chaperones Hsc70 and Hsp110, the ThT fluorescence measurements continued with the other aggregation-prone peptide Aβ42. These data have also been entered into AmyloFit, a free online platform for the processing and analysis of protein aggregation kinetic data

(https://www.amylofit.ch.cam.ac.uk/login), to generate graphs as presented in Fig. 13.



A) Aβ42 at increasing concentrations 2-5 μM showing that the fibril formation is dependent on the concentration of Aβ42monomers. ***B)*** *Aβ42 at 4 μM concentration and with 0.05 or 0.5 μM DNAJB6 showing suppression of fibril formation. Data from experiment 200427.*

Half times of Aβ42 can be taken from AmyloFit or calculated in Excel as average of triplicates, or duplicates where indicated.

3.4.1 The effect of DNAJB6 and Hsc70/Hsp110/ATP on the fibril formation by Aβ42

The first experiments were performed in with 200 mM HEPES buffer as used in the experiments with polyQ. results are presented in Table 7. Compared to polyQ Aβ42 is even more aggregation-prone and all t1/2-values were much lower than for polyQ Table 7.

Table 7. ***Aβ42 in absence and presence of DNAJB6 and Hsc70/Hsp110/ATP in 200 mM HEPES buffer.*** *The average $t_{1/2}$ of triplicate samples with 2, 3, 4 and 5 µM Aβ42 in 200 mM HEPES/10 mM NaPB at pH 8.0 with or without salts (=NaCl 150 mM and $MgCl_2$). Data from experiment 200415. HHA=Hsc70/Hsp110/ATP*
nd =not determined

	no JB6	**SE**	**0.05 uM JB6**	**SE**	**0.5 uM JB6**	**SE**
No Hsc70/Hsp110/ATP						
2 µM AB	0.5 h	0.1	0.8 h	0.03	7.0 h	2.2
2 µM AB salts	nd	nd	nd	nd	0.5 h	0.1
3 µM AB	0.5 h	0	0.7 h	0	5.0 h	1.4
4 µM AB	0.5 h	0	0.7 h	0	4.0 h	0.3
5 µM AB	0.5 h	0	0.7 h	0.1	1.8 h	0.1
5 µM AB salts	0.2 h	0	0.5 h	0	1 h	0.1
With Hsc70/Hsp110/ATP						
2 µM AB	0.7 h	0.1	1.7 h	0.4	0.5 h	2.6
2 µM AB salts	nd	nd	nd	nd	0.5 h	0.1
3 µM AB	0.7 h	0.1	1.9 h	0.5	3.5 h	0.5
4 µM AB	0.7 h	0	1.3 h	0.3	3.1 h	0.7
5 µM AB	0.7 h	0.1	1.1 h	0.1	2.2 h	1.3
5 µM AB salts	0.5 h	0.1	0.8 h	0.03	1 h	0.1

However, compared to previous measurements with DNAJB6 all t1/2-values were very low (approximately t1/2 = 0.5h) and not very different between 2, 3, 4 and 5 µM Aβ42. Moreover, the suppression effect by DNAJB6 was not very pronounced (t1/2=5 h). We decided to repeat the experiment under conditions with lower ionic strength as previously used (12).

Since the buffer with 200 mM HEPES was chosen in order to avoid a pH change by addition of 5 mM ATP we instead adopted another approach, namely to set the pH of the ATP stock solution to pH 8 before using it for addition to 20 mM NaPB pH 8.0 in the next experiment. The results are presented in Table 8.

Table 8. ***Aβ42 in absence and presence of DNAJB6 and in absence and presence of Hsc70/Hsp110/ATP in 20 mM NaPB buffer.*** *The average $t_{1/2}$ values of Aβ in 20 mM NaPB at pH 8.0. Data from experiment 200427.*
HH = Hsc70/Hsp110
nd =not determined

			No ATP			
$t_{1/2}$	**only buffer**	**SE**	**0.05 µM JB6**	**SE**	**0.25 µM JB6**	**SE**
2 µM AB	1.5 h	0	10 h	0.3	35 h or more	nd
3 µM AB	1.0 h	0.1	5 h	0.9	34 h or more	nd
4 µM AB	0.7 h	0.1	4 h	0.8	21 h	2.7
5 µM AB	0.6 h	0.1	1.3 h	0.3	10 h	0.8
			With 2.5mM ATP			
2 µM AB	1.0 h	0.1	4.0 h	0.5	nd	nd
3 µM AB	1.0 h	0	2.0 h	0.5	nd	nd
4 µM AB	0.7 h	0.03	0.9 h	0.05	nd	nd
5 µM AB	0.6 h	0.1	0.7 h	0.1	nd	nd
2 µM AB HH	3.0 h	0.3	35 h	nd	50 h or more	nd
3 µM AB HH	2.0 h	0	11 h	2.5	40 h or more	nd
4 µM AB HH	1.3 h	0.1	18 h	6.9	30 h	1.7
5 µM AB HH	1.1 h	0.05	5.5 h	1.4	14 h	3.2

In this experiment, we note that:

(i) The $t_{1/2}$ values decrease with increasing concentration of Aβ42, as expected, and increase with addition of DNAJB66, as expected. The addition of 0.05 µM DNAJB6 increased the $t_{1/2}$ with approximately a factor 5 (from 1h to 5h at 3 µM Aβ42). When adding 0.25 µM DNAJB6 the $t_{1/2}$ values increased even further with $t_{1/2}$ values > 35h.

(ii) In presence of DNAJB6 the $t_{1/2}$ values were markedly decreased by the addition of ATP (from 5h to 2h at 3 µM Aβ42), yet ATP in itself had no significant effect on $t_{1/2}$ values of Aβ42.

(iii) In presence of DNAJB6 and Hsc70/Hsp110/ATP all $t_{1/2}$ values were much increased (t1/2 = >50 h, >40 h, 20 h and 14 h for 2, 3, 4, and 5 µM Aβ42 respectively.

The last observation (iii) is also clearly seen in the ThT fluorescence assays curves, which is presented in Fig. 14 and Fig. 15.

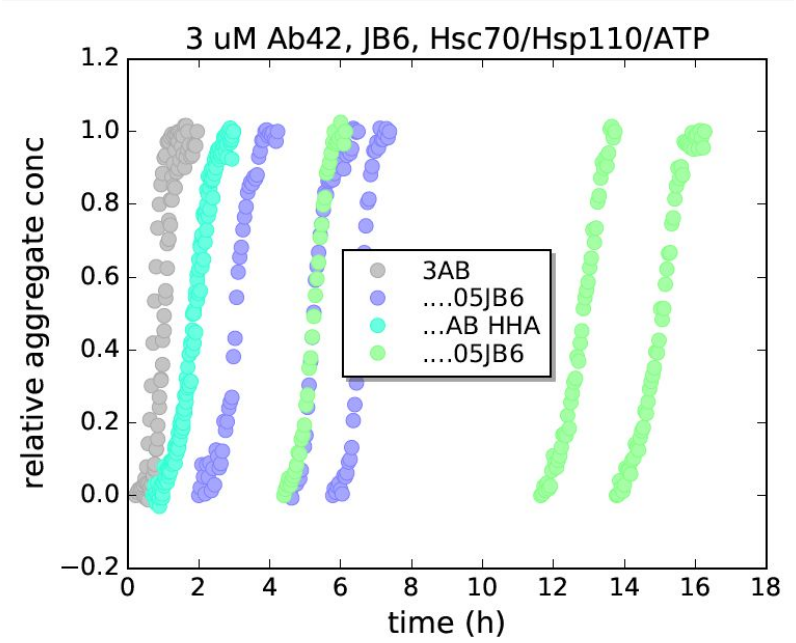


Fig. 14. ***The interaction of Hsc70/Hsp110/ATP with DNAJB6.*** *Image created in Amylofit with data from 200427.*

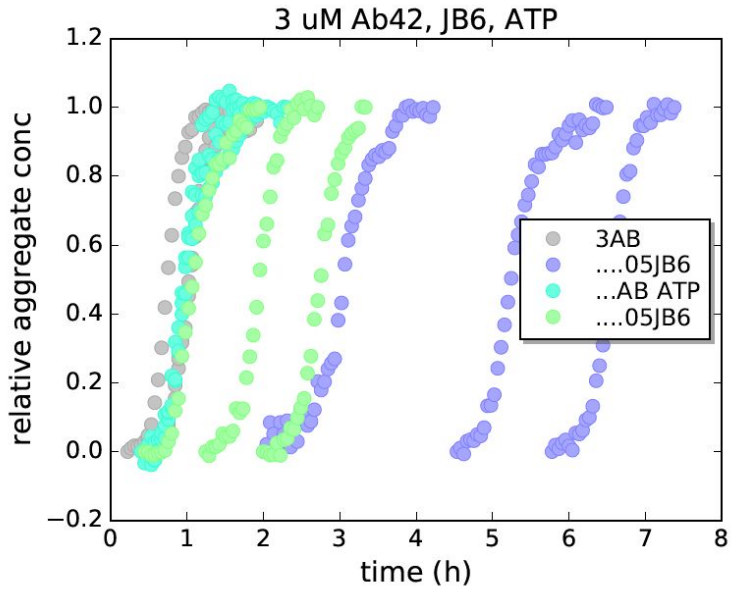


Fig. 15. ***The effect of ATP on DNAJB6.*** *Image created in Amylofit with data from 200427.*

3.4.2 The effect of DNAJB6 and adding Hsc70/ATP without Hsp110 on the fibril formation by Aβ42

In the final experiments there is no Hsp110 present since we ran out of this chaperone and we recorded data to explore the effect of ATP with only Hsc70 and DNAJB6 in various combinations, as presented in

Table 9.

Table 9. ***Effect of ATP with only Hsc70 and DNAJB6 in various combinations.*** *The average $t_{1/2}$ values of Aβ in 20 mM NaPB pH 8.0 ± 0.05 uM DNAJB6, ±ATP and ±0.2 uM Hsc70. Data from experiment 200429*
‘ = singlicates
nd = not determined

$t_{1/2}$	only buffer	SE	2.5 mM ATP	SE	0.05 µM JB6	SE	2.5 mM ATP 0.05 µM JB6	SE
2 µM AB	1.3 h	0.02	1.3 h	0.1	11 h	1.9	5 h	0.3
3 µM AB	1.0 h	0.03	0.8 h	0.05	14 h	1.2	1.8 h	0.4
4 µM AB	0.8 h	0	0.7 h	0.03	4.5 h	0.4	1.1 h	0.07
5 µM AB	0.4 h	0.03	0.4 h	0	1 h’	nd	0.8 h	0.08
2 µM AB 0.2 µM Hsc70	32 h	1.1	31 h	5.6	1.8 h	0.03	6.7 h	0.3
3 µM AB 0.2 µM Hsc70	18 h	5.6	21 h	1.4	1.3 h	0.05	2.1 h	0.07
4 µM AB 0.2 µM Hsc70	4.4 h	0.5	14 h	2.0	1.1 h	0.03	2.1 h	0.4
5 µM AB 0.2 µM Hsc70	1.4 h	0.2	8.4 h	3.1	0.9 h	0.05	1.6 h	0.4

According to the $t_{1/2}$ values in Table 9, we observe that

(i) ATP has no effect on the t1/2-value for Aβ42 alone

(ii) DNAJB6 increases the t1/2-values, from 1.3 h to 11 h (8.5-fold) for 2 µM Aβ42, from 1.0 h to 14 h (14-fold) for 3 µM Aβ42, from 0.8 h to 4.5 h (5.6-fold) for 4µM Aβ42 and from 0.4 to 1 h (2.5-fold) for 5µM Aβ42, as expected

(iii) ATP pronouncedly decreased the t1/2-values in presence of DNAJB6 to half or less than half of the value (e.g. from 4.5 h to 1.1 h at 4μM Aβ42).

(iv) 0.2 μM Hsc70 increased the t1/2 values, as much as DNAJB6, with much the same values in presence or absence of ATP (e.g. from t1/2=1.0 h to 18 h at 3μM Aβ42) but when 0.05 μM DNAJB6 is also present the t1/2 values are instead decreased (from 14h to 2.1h at 4μM). There is also an even greater decrease of t1/2 when DNAJB6 is present with Hsc70 (from 18h to 1.3h at 4μM).

The experiment in Table 9 was repeated, and also two different batches of DNAJB6 were tested (the batch so far used in all experiments, opt 5, and another one, opt 1) and the result is presented in Table 10.

Table 10. ***Effect of ATP with only Hsc70 and DNAJB6 in various combinations in different DNAJB6-preparations.*** *The average $t_{1/2}$ values of Aβ in 20 mM NaPB pH 8.0 ± 0.05 uM DNAJB6, ±ATP and ±0.2 uM Hsc70 and two different batches of DNAJB6, opt 5 and opt 1. Data from experiment 200504*
nd = not determined
‘ = singlicates

$t_{1/2}$	only buffer	SE	5 mM ATP	SE	0.05 μM JB6 opt5	SE	0.05 μM JB6 opt1	SE
2 μM AB	1.7 h	0.05	1.3 h	0.03	17 h	3.1	nd	nd
3 μM AB	1.3 h	0.03	1.0 h	0	9.3 h	1.7	17 h	0.5
4 μM AB	1.0 h	0.03	0.7 h	0	4.8 h	1.6	12 h	1.9
5 μM AB	0.8 h	0.05	0.6 h	0	3.3 h	0.8	11 h	0.9
2 μM AB Hsc70	48 h or more	nd	2.3 h	0.07	6.2 h	0.2	11 h’	nd
3 μM AB Hsc70	9.7 h	1.1	1.4 h	0.03	3.8 h	0.03	9.0 h	0.1
4 μM AB Hsc70	5.7 h	0.7	1.1 h	0.03	2.5 h	0.6	5.3 h	0.3
5 μM AB Hsc70	3.5 h	0	0.8 h	0.03	1.6 h	0.2	3.3 h	1.0

When comparing the $t_{1/2}$ values of DNAJB6 opt1 with opt5 from Table 10, opt1 seems to have a greater increasing effect on $t_{1/2}$ values of Aβ42 (from 1.3 h to 9.3 h for opt5 and to 17 h for

opt1 at 3μM) showing that there may be batch-to-batch differences between the DNAJB6-preparations

3.4.3 Comparing the effect of DNAJB6 and Hsc70 and the effect of ATP and ADP respectively for DNAJB6 WT and DNAJB6 F91L mutant.

In the previous experiment Hsc70 seemed to have a greater increasing effect on the $t_{1/2}$ values of Aβ42 than DNAJB6 but the concentration of Hsc70 (0.2 μM) was higher than of DNAJB6 (0.05 μM). In the next experiment, an equal amount of DNAJB6 and Hsc70 were added and the effect of ATP and ADP, respectively was compared, and the results are presented in Table 11.

Table 11. ***Comparing equal amounts of DNAJB6 and Hsc70 separately and in combination in response to ATP and ADP.*** *Aβ42 with ATP/ADP, DNAJB6 and Hsc70. The average $t_{1/2}$ values of 2, 3 and 4 μM Aβ42 with 5 mM ATP/ADP, 0.2 μM DNAJB6 and 0.2 μM Hsc70. 2 and 3 μM Aβ42 are performed as triplicates and 4 μM Aβ42 are duplicates. Data from experiment 200508.*
∞ = no increase in fluorescence indicating no fibril formation during the experimental time
'' = duplicates

$t_{1/2}$	buffer	SE	JB6	SE	Hsc70	SE	JB6, Hsc70	SE
2 Aβ No ATP/ADP	1.4 h	0.03	∞		∞		∞	
2 Aβ ATP	1.2 h	0	∞		2.1 h	0.2	∞	
2 Aβ ADP	1.3 h	0.03	∞		∞		30 h	2.4
3 Aβ No ATP/ADP	1.0 h	0	18 h'' or more		7.5 h''	0.3	∞	
3 Aβ ATP	0.8 h	0.03	7.0 h	0.7	1.2 h	0.03	9.6 h''	0.1
3 Aβ ADP	0.8 h	0.1	∞		2.2 h	0.1	∞	
4 Aβ No ATP/ADP	0.7 h''	0	15 h''	2.9	2.4 h''	0.1	20 h''	0.3
4 Aβ ATP	0.6 h''	0	3.7 h''	0.9	1.0 h''	0.1	7.5 h''	0.03
4 Aβ ADP	0.7 h''	0.03	10 h''	0.9	1.2 h''	0.05	∞''	

A comparison of DNAJB6 and Hsc70 shows that that 0.2 μM DNAJB6 gave a greater increase in $t_{1/2}$ values than 0.2 μM Hsc70 (from 0.7h to 15h/2.4h JB6/Hsc70) whereas a combination of the two chaperones give the largest $t_{1/2}$-value (20 h). In absence of any chaperone there were no major effects of ATP and ADP on the t1/2-value of fibril formation by Aβ42 itself. In presence of 0.2 μM DNAJB6 there are decreased in $t_{1/2}$ values by ATP or ADP, and the decrease when adding ATP is greater than when adding ADP (for example from 15 h to 3.7 h/10 h ATP/ADP at 4μM Aβ42). In presence of 0.2 μM Hsc70 there were also but somewhat less pronounced decreased t1/2-values by added ATP/ADP (for example from 7.5 h to 1.2 h/2. 2h ATP/ADP at 3μM Aβ42). In presence of both DNAJB6 and Hsc70 the data on the ATP/ADP-differences are more complex to interpret. In presence of ATP the same trend is observed that DNAJB6 increased $t_{1/2}$-values more than Hsc70 and a combination of the two chaperones gave the largest $t_{1/2}$-value, although not as large as in absence of ATP. However, all data with 4μM Aβ42 are only duplicates instead if triplicates so the average values could be somewhat less accurate.

The experiment was repeated to collect more observations on the effects of ATP and ADP, and also the mutant DNAJB6 F91L was included to see of it responded differently than DNAJB6WT and the results are presented in Table 12.

Table 12. ***Comparing equal amounts of DNAJB6 and Hsc70 separately and in combination in response to ATP and ADP in DNAJB6 WT and the mutational variant F91L.*** *Aβ42 with DNAJB6 WT/F91L, ATP/ADP and Hsc70. The average $t_{1/2}$ values of 2, 3 and 4 μM Aβ42. 2 and 3 μM Aβ42 were performed as triplicates and 4 μM as duplicates. Data from experiment 200511.*
∞ = no increase in fluorescence indicating no fibril formation during the experimental time
‘ = singlicates

$t_{1/2}$	buffer	SE	JB6	SE	F91L	SE
2 Aβ ATP	1.2 h	0.05	12 h	0.03	32 h’	
2 Aβ ADP	1.3 h	0.08	∞		∞	
3 Aβ ATP	0.8 h	0	5.2 h	0.6	∞	
3 Aβ ADP	0.9 h	0.1	7.1 h	2.8	∞	
4 Aβ ATP	0.7 h	0	1.5 h	0.2	24 h’	
4 Aβ ADP	0.7 h	0	7.1 h	0.6	∞	
			With Hsc70			
2 Aβ ATP	1.9 h	0.1	∞		28 h’	
2 Aβ ADP	∞		31 h	2.9	16 h	3.3
3 Aβ ATP	1.2 h	0.1	7.7 h	0.7	∞	
3 Aβ ADP	1.7 h	0.2	39 h’’	3.2	33 h	3.8
4 Aβ ATP	1.0 h’’	0.03	6.5 h’’	0.2	∞	
4 Aβ ADP	1.2 h’’	0.03	13 h’’	0.6	∞	

Like in Table 11, the $t_{1/2}$ values of Aβ42 with ATP and ADP were approximately equal (0.8 h with ATP and 0.9 h with ADP at 3μM Aβ42) in Table 12. The other data in Table 12 essentially confirm the impression that the observation in Table 11, that in presence of

DNAJB6, and with the combination of DNAJB6 and Hsc70, added ATP results in lower t1/2-values than ADP, for both DNAJB6 WT and the mutant F91L.

With 0.2 μM Hsc70 there is no difference in $t_{1/2}$ values between ATP and ADP (1.2 h with ATP and 1.7 h with ADP at 3μM Aβ42). With 0.2 μM DNAJB6 WT, the $t_{1/2}$ values are lower with ATP than with ADP (1.5 h with ATP and 7.1 h with ADP at 4μM Aβ42). With 0.2 μM Hsc70 and 0.2 μM DNAJB6 WT the $t_{1/2}$ values are lower with ATP than with ADP (6.5 h with ATP and 13 h with ADP at 4μM Aβ42).

4.0 Discussion

4.1 On the trimeric chaperone system DNAJB6/Hsc70/Hsp110 and fibril formation by the aggregation-prone peptides polyQ and Aβ42

The aim of this project is to make a first study of the trimeric chaperone system of DNAJB6/Hsc70Hsp110 and ATP and the effects on the fibril formation by the aggregation-probe peptides polyQ and Aβ42 and I will summarize and comment on the findings first with polyQ, then with Aβ42.

4.2 Minor effects of Hsc70, Hsp110 and ATP on the DNAJB6 suppression of fibril formation by polyQ

The $t_{1/2}$ values of 10 μM polyQ were measured in assay buffers with and without additional salts (150mM NaCl or 150mM NaCl and 5 mM $MgCl_2$). The salts slightly increased the $t_{1/2}$ values of polyQ in NaPB, HEPES and NaPB/HEPES (from 5h w/o salts to 6h w NaCl/$MgCl_2$ at 10 μM polyQ in NaPB, see Table 2). This contrasts the situation with Aβ42, where salts had a pronounced effect on the $t_{1/2}$ values (see below). The effects of ion strength on $t_{1/2}$ values of polyQ is unclear, but the amino acid sequence polyQ-peptide, 52 amino acids including 45 Q-residues GAMKSF(Q)45F (2), is chemically very different compared to the 43 amino acids in recombinantly expressed Aβ42 peptide, with the sequence MDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA (12). Whereas the Aβ42 has a number of charged amino acid residues that would respond to changes in electrostatic interactions and vary with ionic strength, the polyQ peptide has only one charged lysine residue.

With polyQ the presence of DNAJB6 increased the $t_{1/2}$ values, which have been seen previously (2). Already at the ratio of 1:0.01 polyQ to DNAJB6 there is a 25% increase of the $t_{1/2}$ value. Thus, DNAJB6 acts at sub-stoichiometric molar ratio, and only a hundredth as much DNAJB6 as polyQ can suppress fibril formation by the polyQ peptide. This study was initiated by and based on previous studies which described suppression of polyQ fibril formation by a trimeric chaperone system of DNAJB1, Hsc70, Hsp110 and ATP (13) (14). DNAJB1 is a human homologue to human DNAJB6, however they are still quite different. Only DNAJB6 contains the S/T-residues that are crucial for function (12). The molar ratio was 1:3 for polyQ to DNAJB1 to show the suppression of the homolog DNAJB1 (13), whereas the ratio of polyQ to DNAJB6 used here is 1:0.1 and 1:0.01. Thus, by far less DNAJB6 than DNAJB1 is required for effect.

4.3 The trimeric chaperone system with DNAJB6, Hsc70, Hsp110 and ATP can enhance the suppression of fibril formation by Aβ42

When the supply of polyQ-peptide that we used (Table 2-6) to compare effects of DNAJB6 with previously published data with DNAJB1 (13) was finished, we turned to our other aggregation-prone peptide, Aβ42. The fibril formation by Aβ42 shows much shorter $t_{1/2}$ values than polyQ; $t_{1/2}$ value of 5 μM Aβ42 is 0.6 h (Table 8) and the $t_{1/2}$ value of 10 μM polyQ is 5 h (Table 2), both in 20 mM NaPB. This suggests that Aβ42 is more prone to aggregate than polyQ, which is also expected from previous data (12) and, as also expected, the $t_{1/2}$ values of Aβ42 decreased with increased concentration, from 1.5 h at 2μM to 0.6h at 5μM (Table 8). The aggregation rates increase with higher concentrations since the number of Aβ42 peptide monomers is then larger per volume unit and subsequently the probability of collisions increases per time unit.

The experiments with Aβ42 were performed under conditions of low ionic strength, in 20 mM NaPB buffer and no salts, since the initial finding that at high ionic strength the aggregation was much faster such that the expected differences in aggregation rate were not seen in 200 mM HEPES and the suppression effect of DNAJB6 was less pronounced (Table 7). The ionic strength effects on Aβ42 were expected, however, it differs from what we observed in case of polyQ, where there were minor effects of ion strength differences. DNAJB6 suppressed fibril formation by Aβ42 and by polyQ, with a factor 10-20 and a factor 3-5, respectively. Overall, it appears as if DNAJB6 has a greater suppressing effect on fibril formation by Aβ42 compared to polyQ.

Importantly, the data obtained with Aβ42 show very clear effects by the trimeric chaperone system with DNAJB6, Hsc70, Hsp110 and ATP (Table 8). The by far largest effect in terms of suppression of fibril formation by Aβ42 was obtained with 0.25 μM DNAJB6 and Hsc70/Hsp110/ATP, with $t_{1/2}$ values >50 h, >40 h and 30 h, and 14 h for 2, 3, 4, 5 μM Aβ42. This should be compared to corresponding $t_{1/2}$ values for 2, 3, 4, 5 μM Aβ42 without chaperones (1.5 h, 1 h, 0.7 h and 0.6 h), and corresponding $t_{1/2}$ values for 2, 3, 4, 5 μM Aβ42 with 0.25 μM DNAJB6 (>35 h, > 34 h, 21 h and 10 h). Thus, even if already DNAJB6 itself has a remarkably good effect in suppression of fibril formation the effect is further enhanced in the trimeric chaperone system with DNAJB6, Hsc70, Hsp110 and ATP, at all concentrations of Aβ42. In conclusion, 0.05 μM DNAJB6 alone increased the $t_{1/2}$ values with approximately a factor 5 (from 1h to 5h at 3μM Aβ42) whereas 0.05 μM DNAJB6 in the trimeric chaperone system increased the $t_{1/2}$ values 9 to 26-fold.

An unexpected observation in Table 8 is that the addition of ATP also showed an effect on DNAJB6 only, with decreased $t_{1/2}$ values (from 10 h, 5 h, 4 h and 1.3 h to 4 h, 2 h, 0.9 h and 0.7 h for 2, 3, 4, 5 μM Aβ42). This is not due to direct effects of ATP on the aggregation of Aβ42 since the $t_{1/2}$ values for 2, 3, 4, 5 μM Aβ42 are not much different in absence/presence of ATP (1.5/1 h, 1/1 h, 0.7/0.7 h and 0.6/0.6 h). The unexpected effect of ATP in decreasing

the $t_{1/2}$ values for fibril formation by Aβ42 may be due to a contamination of Hsc70 in the DNAJB6 preparation and is further discussed in the following where the direct effects of the nucleotides (ATP, ADP and non-hydrolyzable ATP-analogue) on DNAJB6 and Hsc70, respectively, is discussed in terms of the data shown in Tables 9-12.

Thus, with Aβ42 there was an enhancement of the DNAJB6 suppression of fibril formation in the trimeric chaperone system, although no such enhancement was detected with polyQ. It is not clear why there is a difference between Aβ42 and polyQ. It is possible that one simply needs to use larger amounts of DNAJB6 with polyQ than with Aβ42. Here we only used one tenth (1/10) as much DNAJB6 as polyQ peptide, contrasting the study that previously described the trimeric chaperone system with a homologue to DNAJB6, DNAJB1 (13), where the molar ratios were indeed reversed: a 3-fold surplus of DNAJB1 in relation to polyQ. Another possibility could be that the high ionic strength used in most of our experiments could, although it does not affect the aggregation rate of polyQ peptide in itself, could have weakened the electrostatic interactions supposedly required for interaction between Hsc70 and DNAJ-proteins (8).

4.4 ATP effects and DNAJB6 in the trimeric chaperone system

When we ran out of Hsp110 we continued experiments without Hsp110. The Hsp110 chaperone is a NEF (nucleotide exchange factor) and its role is to promote the turnover of Hsc70 by speeding up the release of ADP after hydrolysis (7), (13), (14). Without Hsp110 we focused on collecting data on the direct effects of ATP on DNAJB6 and Hsc70 in different combinations, to compare the effects of DNAJB6 and Hsc70 alone and to evaluate the difference in effect of ATP and the nucleotide variants ADP and non-hydolyzable ATP-analogue (Table 9-12).

When DNAJB6 and Hsc70 separately were added to Aβ42 they increased the $t_{1/2}$-values, i.e. suppressed the fibril formation, to quite similar extent. However, in contrast to this, with added ATP the $t_{1/2}$-values in presence of DNAJB6 decreased to half or less than half of the value. When both DNAJB6 and Hsc70 were present ATP decreased the $t_{1/2}$ values (Table 9). Thus, when each of the two chaperones worked alone, they yielded relatively high $t_{1/2}$ values (18 h with Hsc70 and 14 h with DNAJB6 at 3μM Aβ42), but when added together in presence of ATP the $t_{1/2}$ values were low (t1.3 h at 3 μM Aβ42).

ATP and ADP showed no effect on $t_{1/2}$ values of the aggregation by Aβ42 itself, but they affected the $t_{1/2}$ values when the chaperones were present (Table 11-12). In presence of DNAJB6, and with the combination of DNAJB6 and Hsc70, the $t_{1/2}$-values were repeatedly lower with ATP than ADP.

To summarize, in this study we have obtained data showing that there is an enhancement of DNAJB6 suppression of fibril formation by Aβ42 in the trimeric chaperone system. The $t_{1/2}$ values for fibril formation are increased in presence of DNAJB6/Hsc70/Hsp110/ATP compared to the $t_{1/2}$ values obtained with DNAJB6 only (Table 8), which is also clearly seen

in the ThT fluorescence assay curves (Fig. 14Fig. 15). This suggest that the process is concentration-dependent, with 0.25 μM DNAJB6 resulting in even better suppression than 0.05 μM DNAJB6. The data obtained in this study therefore implies that there is an interaction between Hsc70 and DNAJB6, such that DNAJB6 can interact with and hand over the substrate to Hsc70, as suggested previously for the homologue DNAJB1 (7), (13). We were also surprised to find some effects of ATP on DNAJB6, since this is a chaperone without nucleotide binding site, and it is possible that the preparation of DNAJB6 is contaminated with Hsc70 and that ATP affects the Hsc70 instead of DNAJB6. It is commonly known that recombinantly expressed proteins contain varying amounts of Hsc70 since the Hsc70 is chaperoning and assisting in folding of expressed proteins.

How to interpret the experimental observation that in presence of Hsp110 there was an enhancement of the DNAJB6 suppression of fibril formation by Aβ42 (increased $t_{1/2}$ values, Table 8) but without Hsp110 there was no enhancement and instead there were decreased $t_{1/2}$-values (Tables 9-12)? Since the Hsp110 chaperone is a NEF (nucleotide exchange factor) and its role is to promote the turnover of Hsc70 by speeding up the release of ADP after hydrolysis (7), (13), (14) we speculate that the long $t_{1/2}$ values observed with the trimeric chaperone system is the result of continuous capture of Aβ42 oligomers (15), that function as nuclei for fibril formation, which are perhaps dissolved during ATP-hydrolysis and subsequently released from the trimeric chaperone system as Aβ42 monomers (Fig. 16). It is known that when DNAJB6 works alone it binds strongly to the oligomeric forms of the Aβ42 peptides (15), such that they are not free in solution to form fibrils. This can explain why DNAJB6 itself has a certain capacity to suppress fibril formation as long as there is DNAJB6 enough for irreversible binding of these Aβ42 oligomers, that only make up a few % of the total population of Aβ42 peptides. In the absence of Hsp110/NEF there would be no, or much slower, ATP-hydrolysis and that in turn could explain why instead Hsc70 and ATP could instead release the bound Aβ42 oligomers, without dissolving them into Aβ42 monomers, which would result in speeding up the fibril formation and thereby decreasing the $t_{1/2}$ values, just as observed in Tables 9-12. It is known that Hsc70 has a low affinity substrate binding conformation in the presence of ATP, and a high affinity substrate binding conformation in the presence of ADP (7), which could result in more release of substrates when ATP is present to when ADP is present (Table 11 and 12).

Importantly, the data obtained in this study provides the first experimental evidence that there is an interaction between Hsc70 and DNAJB6 where DNAJB6 hands over the substrate to Hsc70. Future experiments to further investigate this phenomenon would be to explore if it works also with polyQ if using a higher concentration of DNAJB6 and a lower ionic strength, to supply ATP continuously on a longer time-scale by providing phosphoenolpyruvate and pyruvate kinase and to evaluate the outcome with a known DNAJB6-mutant (H31Q) that cannot bind to Hsc70 due to a mutation in the HPD-motif.

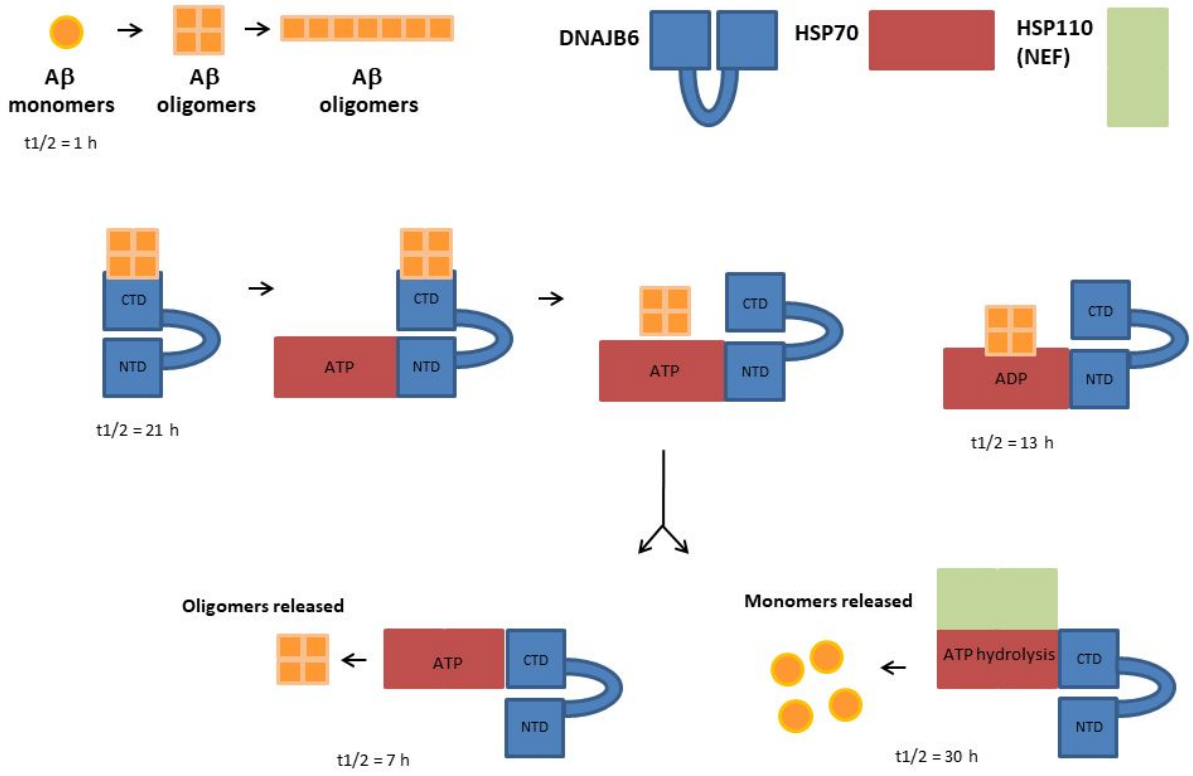


Fig. 16. ***Possible interpretation of obtained data.*** *Image created in PowerPoint by Cecilia Emanuelsson.*

5.0 Conclusions

1. DNAJB6 can suppress fibril formation by Aβ42 in the trimeric chaperone system at very low molar ratio.

Data obtained in this project provide the first experimental evidence that DNAJB6 can suppress fibril formation, as previously found for the homologue DNAJB1 (13), in a trimeric chaperone system with DNAJB6, Hsc70and Hsp110. The molar ratio of peptide to DNAJB6 was as low 1:0.01 Aβ42 to DNAJB6, whereas for the molar ratio of peptide to DNAJB1 was much higher, 1:3.

2. The interactions in the trimeric chaperone system are ATP-hydrolysis-dependent.

The $t_{1/2}$ value for fibril formation by Aβ42 was increased for DNAJB6 in the trimeric system compared to DNAJB6 alone. In the absence of the nucleotide exchange factor Hsp110 the $t_{1/2}$ values were instead very low, thus, the suppression of Aβ42 fibril formation is dependent on ATP-hydrolysis.

3. DNAJB6 may hand-over substrate to Hsc70.

Hsc70, Hsp110 and ATP without DNAJB6 did not significantly affect the $t_{1/2}$ values of fibril formation by Aβ42, but in the presence of DNAJB6 the $t_{1/2}$ values are strongly increased. This suggests an interaction where substrate is transferred from DNAJB6 to Hsc70.

This is a short answer to the question “Can DNAJB6 hand over its substrate, such as polyQ or Aβ42, to Hsc70?”. An even shorter answer would be “yes”. At least to Aβ42 and at least under the tested conditions in this project.

4. DNAJB6 and Hsc70 may release the captured oligomers of Aβ42.

In the presence of ATP, a dimeric chaperone system of DNAJB6 and Hsc70 without the nucleotide exchange factor Hsp110, gave very low $t_{1/2}$ values. A possible explanation could be that Hsc70 may release Aβ42 oligomers bound to DNAJB6.

6.0 Acknowledgments

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Lastly, I would like to thank my family and friends for loving me and always being there for me. I could not have come this far if it were not for you. And a big thanks to my boyfriend for giving me motivation every day.

7.0 References

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Appendix

Appendix 1

Wells	1	2	3	4	5	6	7	8	9	10	11	12
A	2 ABATP	2 ABH/ATP	2 ABADP	2 ABH/ADP	2 ABATPJB6	2 ABH/ATPJB6	2 ABADPJB6	2 ABH/ADPJB6	2 ABATPF91L	ABH/ATPF91L	2 ABADPF91L	ABH/ADPF91L
B	2 ABATP	2 ABH/ATP	2 ABADP	2 ABH/ADP	2 ABATPJB6	2 ABH/ATPJB6	2 ABADPJB6	2 ABH/ADPJB6	2 ABATPF91L	ABH/ATPF91L	2 ABADPF91L	ABH/ADPF91L
C	2 ABATP	2 ABH/ATP	2 ABADP	2 ABH/ADP	2 ABATPJB6	2 ABH/ATPJB6	2 ABADPJB6	2 ABH/ADPJB6	3 ABH/ADPJB6	ABH/ATPF91L	2 ABADPF91L	ABH/ADPF91L
D	3 ABATP	3 ABH/ATP	3 ABADP	3 ABH/ADP	3 ABATPJB6	3 ABH/ATPJB6	3 ABADPJB6	2 ABATPF91L	3 ABATPF91L	ABH/ATPF91L	3 ABADPF91L	ABH/ADPF91L
E	3 ABATP	3 ABH/ATP	3 ABADP	3 ABH/ADP	3 ABATPJB6	3 ABH/ATPJB6	3 ABADPJB6	3 ABH/ADPJB6	3 ABATPF91L	ABH/ATPF91L	3 ABADPF91L	ABH/ADPF91L
F	3 ABATP	3 ABH/ATP	3 ABADP	3 ABH/ADP	3 ABATPJB6	3 ABH/ATPJB6	3 ABADPJB6	3 ABH/ADPJB6	3 ABATPF91L	ABH/ATPF91L	3 ABADPF91L	ABH/ADPF91L
G	4 ABATP	4 ABH/ATP	4 ABADP	4 ABH/ADP	4 ABATPJB6	4 ABH/ATPJB6	4 ABADPJB6	4 ABH/ADPJB6	4 ABATPF91L	ABH/ATPF91L	4 ABADPF91L	ABH/ADPF91L
H	4 ABATP	4 ABH/ATP	4 ABADP	4 ABH/ADP	4 ABATPJB6	4 ABH/ATPJB6	4 ABADPJB6	4 ABH/ADPJB6	4 ABATPF91L	ABH/ATPF91L	4 ABADPF91L	ABH/ADPF91L

JB6	
F91L	

Buffer:	conc
NaPB pH 8.0	20 mM
no salts	

All samples w DNAJB6 opt 5
HA=Hsp70/ATP
JB6=DNAJB6

Sample	F91L	~~opt 1~~/opt 5
conc	2.2 mg/ml	1.6 mg/ml
conc	81 µM	59 µM

Substance	AB	AB	AB
Stock	10 µM	10 µM	10 µM
Conc in 100 µl	2 µM	3 µM	4 µM
Conc in 50 µl	4 µM	6 µM	8 µM
No. of wells	36	36	24
Vol in 50 µl	20 µl	30 µl	40 µl
Vol in x wells	720 µl	1080 µl	960 µl
Total volume nee	800 µl	1200 µl	1000 µl

	ATP	~~ATP w Na~~	ATP w Na pH 8.0	ADP w Na pH 8.0
conc	128 mM	~~128 mM~~	128 mM	128 mM
molar mass	507.18 g/mol	~~551.14 g/mol~~	551.14 g/mol	427.20 g/mol
aliquots	20	~~15~~	32	>32
V per aliquot	100 µl	~~100 µl~~	50 µl	50 µl/100 µl
mass	130 mg	~~106 mg~~	106 mg	273 mg
water	2.0 ml	~~1.5 ml~~	1.5 ml	5 ml
			and HCl, KOH	and HCl, KOH

3000 µl / 3.0 ml

Prepare this mix seperately

Mix	Hsp70	ATP	ADP	ThT	F91L	DNAJB6 opt 5
Stock	10 µM	128 mM	128 mM	2 mM	10 µM	10 µM
Conc 100 µl	0.2 µM	5 mM	5 mM	20 µM	0.2 µM	0.2 µM
Conc 50 µl	0.4 µM	10 mM	10 mM	40 µM	0.4 µM	0.4 µM
8x50 µl	Volume 500 µl	20.0 µl	39.1 µl	39.1 µl	10.0 µl	20.0 µl	20.0 µl
	Total volume nee	120 µl	240 µl	240 µl	120 µl	80 µl	80 µl

Fig. 17. ***ThT fluorescence assays during 200415-200511.***

Appendix 2

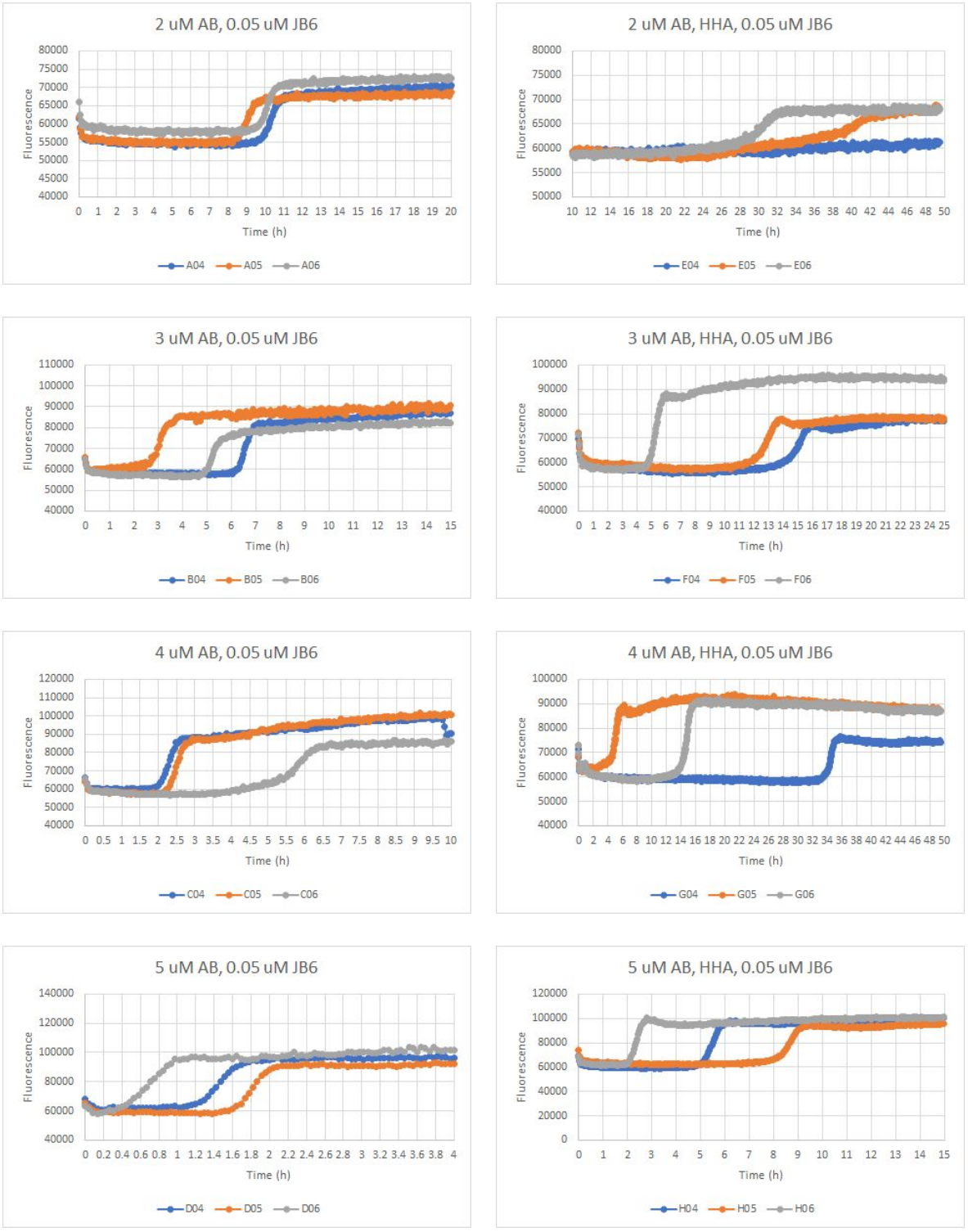

Fig. 18. ***Increased $t_{1/2}$ for Aβ42 with DNAJB6 and Hsc70/Hsp110/ATP - 0.05 µM DNAJB6.*** *Data from experiment 200427, which are represented in Fig. 14 and Fig. 15 as well.*

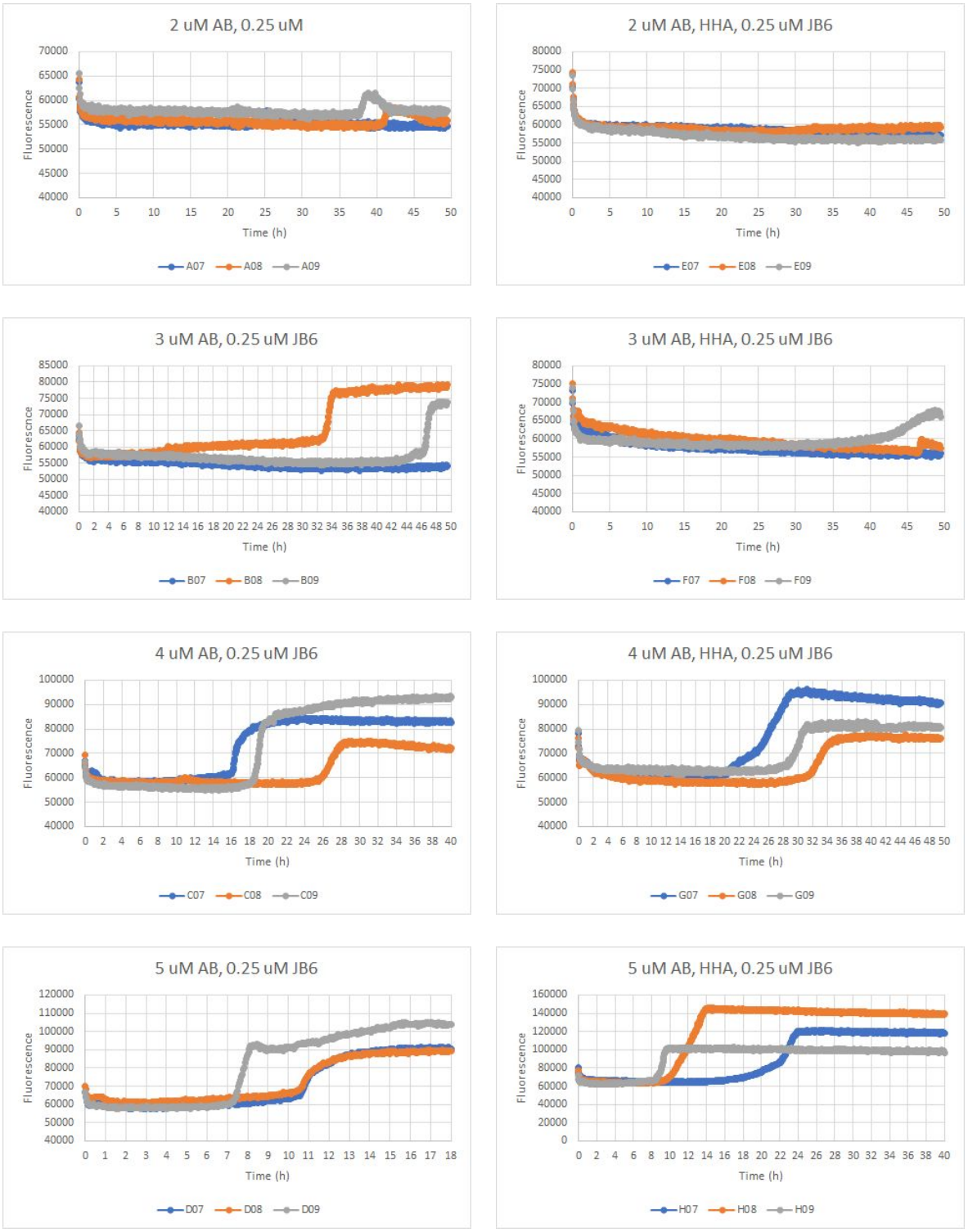

Fig. 19. ***Increased $t_{1/2}$ for Aβ42 with DNAJB6 and Hsc70/Hsp110/ATP - 0.25 µM DNAJB6.*** *Data from experiment 200427, which are represented in Fig. 14 and Fig. 15 as well.*